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XXXI CYCLE

THE MULTIPLE FACES OF THE MUTUAL SUPPORT BETWEEN MCs AND B CELLS IN INFLAMMATION AND CANCER: A COMPLEXITY THAT RESIDES IN THE MICROENVIRONMENT

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1. ABSTRACT

Mast cells (MCs) are long living cells widely distributed in mucosal and connective tissues, in close contact with blood vessels and the external environment. They are known for their predisposition to cross-talk with many cell types and in this work the mutual interplay between MCs and B cells has been object of interest. We focused our attention on the different MCs' and B cells' subsets that can communicate in the peritoneal cavity and the intestine: serosal and mucosal subtypes of MCs and B-1 and B-2 lineages of B lymphocytes. We uncovered that B-2 cells require MCs' contribution for their activation but fail to significantly impact MC's behaviour. On the other hand, B-1 cells do not need MCs to survive and achieve their functions, but exert an intriguing effect exacerbating the activation of MCs. Specifically, B-1 cells induce an increase in the synthesis and release of pro-inflammatory mediators from IgE/Ag activated MCs and enhance their membrane expression of ST2, the receptor for the IL-33. This cytokine is an early danger-sensing molecule important in the activation of MCs in the pathological contexts studied in this work: the intestinal acute inflammation and colorectal cancer (CRC).

MC-B cell interaction was then followed through *in vivo* analyses: the distribution of MCs was investigated in a B-1-deficient mouse model while B cells were characterized in MCs-lacking mouse models both in physiological condition and in the DSS-induced acute intestinal inflammation. The density of MCs resulted to be increased in the lungs and in the small intestine of the *bumble* B-1-deficient mouse model studied in physiological conditions. On the contrary, the distribution of B cell subsets and B cells' markers of activation were not altered in MCs-deficient mice. Differently, we were able to prove that the presence of MCs is important for the regulation of IgA responses in homeostasis and that MCs support the IgA skewing during the intestinal inflammation.

Since chronic gut inflammation predisposes to the onset of CRC, and that the distribution and activation of both MCs and B cells are dramatically altered in this pathology, we explored whether MCs could influence the behaviour of B lymphocytes also in this context. In the subcutaneous CRC mouse model studied in this work, B cells infiltrate the tumor and accumulate in the tumor-draining lymph nodes (dLNs). We ascribe this observation to an increase of chemotactic factors among which CCL20 and CXCL13. Interestingly, in MC-deficient tumor-bearing mice, reduced tumor-infiltrating B cells and no increase of CCL20 and CXCL13 in dLNs, were observed; moreover in MC-reconstituted tumor-mice the wild type conditions were restored. In light of our data, we propose a model in which MCs' direct interaction with cancer cells promotes the exacerbation of a milieu enriched in

pro-inflammatory factors; among these mediators, the MCs-derived TNF-α turned out to be responsible for the increased induction of CCL20 in cancer cells. This chemotactic factor is of great relevance since it is known both to enhance tumor spreading and to attract immune cell types in inflammatory sites.

Collectively, our results provide novel insights in the MC-B cell cross-talk, emphasising that B-1 cells can support the allergic and inflammatory functions of MCs and, furthermore, for the first time trough *in vivo* experiments, we are able to show that MCs provide valuable support for IgA-dependent humoral functions. During carcinogenesis, instead, the chemotaxis of B lymphocytes into CRC sites is indirectly promoted by the activation of MCs. In conclusion, the specific signals that modify the microenvironment in these pathologies must be carefully considered when analysing MC/B cell interplay in order to design targeted therapies.

2. LIST OF ABBREVIATIONS

Ab: antibody
Ag(s): antigen(s)
AID: activation-induced cytidine deaminase
AOM: azoxymethane
APC: adenomatous polyposis coli
APCs: antigen presenting cells
APRIL: a proliferation-inducing ligand
BAFF: B cell activating factor
BCR: B cell antigen receptor
BMMCs: bone-marrow derived mast cell(s)
Breg(s): regulatory B cell(s)
BTK: Burton's tyrosine kinase
C_H: immunoglobulin heavy chain constant region
CLP: common lymphoid progenitor
CMPs: common myeloid progenitors
CRC: colorectal cancer
CSR: class switch recombination
CTMC(s): connective-tissue MC(s)
DAG: diacylglycerol
DCs: dendritic cells
dLNs: tumor-draining LNs
DSS: dextran sodium sulfate
DT: diphtheria toxin
EAE: autoimmune encephalomyelitis
EBF(1): early B cell factor(1)
EFG: epidermal growth factor
ELISA: enzyme-linked immunosorbent assay
ERK: extracellular-signal-regulated kinase
FDCs: follicular dendritic cells
FGF2: fibroblastic growth factor 2
Flt3L: Fms-like tyrosine kinase 3 ligand
FO: follicular (B cells)
GAGs: glycosaminoglycans
GC: germinal center
GM-CSF: granulocyte–macrophage colony-stimulating factor
GMPs: granulocyte/monocyte progenitors
HSC: hematopoietic stem cells
i.d.: intradermal
i.p.: intraperitoneal
i.v.: intravenous
IBD: inflammatory bowel disease
ICAM-1: intracellular adhesion molecule
ICS: intracellular staining
IECs: intestinal epithelial cells
Ig(s): immunoglobulin(s)
ILC(2)s: (type 2) innate lymphoid cells
iNKT: invariant natural killer T cells
InsP3: inositol-1,4,5-trisphosphate
ITAMs: tyrosine-based activation motifs
K.I.: Knock-in
K.O.: knock-out
LFA-1: lymphocyte function-associated antigen
LNs: lymph nodes
LP: (intestinal) lamina propria
LPS: lipopolysaccharide
LT α1β2: lymphotaxin α1β2
LTs: leukotrienes
MAPKs: activation of the mitogen-activated protein kinases
MC(s): mast cell(s)
MC_C: chymase-positive MCs
MC_{CT}: tryptase- and chymase-positive MCs
MCp: MCs precursors
MCPT: mast cell protease
MC_T: tryptase-positive MCs
MHC: major histocompatibility complex
MLNs: mesenteric LNs
MMC(s): mucosal mast cell(s)
MPPs : multipotent progenitor precursors
mTORC1/2: rapamycin-sensitive mTOR complex1/2
MZ: marginal zone (B cells)
ndLNs: tumor non-draining LNs
NF: newly formed (B cells)
NFAT: nuclear factor of activated T cells
NK: natural Killer T cells
PAF: platelet activating factor
Pax5: paired box protein 5
PC(s): plasma cell(s)
PDMCs: peritoneal cell-derived MCs
PG: prostaglandin
PI3K: phosphatidylinositol 3-kinase
pIgR: polymeric immunoglobulin receptor
PIP3: phosphatidylinositol 3,4,5-triphosphate

PKC: protein kinase C
PLC: phospholipase C
PMCs: peritoneal MCs
RA: rheumatoid arthritis
RAG1/2: recombination-activating gene-1/2
RMB: Red Mast Cell and Basophil
S1P: sphingosine 1-phosphate
SCF: stem cell factor
SCFA: short-chain fatty acids
SHM: somatic hypermutation
SLE: systemic lupus erythematosus
SP: substance P
SCFA: short-chain fatty acids
SHM: somatic hypermutation
SLE: systemic lupus erythematosus
SP: substance P
SYK: spleen tyrosine kinase
T1 or **T2**: transitional 1 or 2 (B cells)
TAM: tumor-associated macrophages
TD: T cell-dependent
TGF-β: transforming growth factor type- β
Th: helper T cells
TI: T cell-independent
TIBs: tumor-infiltrating B cells
TLRs: through toll-like receptors
TME: tumour microenvironment
TNF-α: tumor necrosis factor-alpha
Treg(s): regulatory T cell(s)
VEGF: vascular endothelial growth factor
Wt: wild type

3. INTRODUCTION

3.1. THE MAST CELL: A PECULIAR CELL IN THE IMMUNE SYSTEM

Mast cells (MCs) belong to a unique cell population within the immune system with an ancient evolutionary history, in fact it is estimated that they appeared 450-500 million years ago in the first vertebrates. The presence of this innate immune cell throughout the evolution leads to believe in the existence of a selective pressure favouring their survival and infers that MCs have beneficial and important roles[1]. In 1878 Paul Ehrlich was the first to characterize this cell in humans in close proximity to nerves and blood vessels. Great efforts have been made subsequently towards the understanding of the origin of MCs until the discovery of its hematopoietic multipotent progenitors in the bone marrow[2]. It is currently known that MCs can virtually be found in any body tissue, and that they are more abundant at tissues' barriers where they can exert a role of immune "sentinels" not only in acute but also in chronic infections[3]. Activated MCs undergo a process defined degranulation that is a compound exocytosis that enables MCs to discharge their content very rapidly and efficiently[4]. MCs have been traditionally classified into subtypes according to their granular content and body distribution: chymase- and tryptase-positive MCs or only tryptase-positive MCs and connective tissue- or mucosal-MCs, in humans and rodents respectively. However, more recent considerations overcome this paradigm: MC's phenotype and functions are in fact largely shaped by the microenvironment and there are suggestions that a new classification of MCs is required[5]. Indeed, it is increasingly clear that these cells are characterized by a great phenotypical heterogeneity and functional plasticity[6].

3.1.1. MC's origin, tissue maturation and *in vitro* studies

One atypical feature concerning the development of the MC is that although this hematopoietic cell is produced and committed in the bone marrow, it accomplishes its maturation only in peripheral tissues. MCs origin has been long debated until, bi-potent progenitor cells capable of giving rise to both basophils and MCs precursors (MCp) inside the granulocyte/monocyte progenitors (GMPs), have been described and isolated in mouse spleen[7] as showed in *figure1*. For this process a fine tuning of different transcription factors is required: GMPs expresses C/EBPα and give rise to a

progenitor with MC and basophil-forming capacity, when it downregulates C/EBPα and upregulates of GATA-2, GATA-3 and Hes-1. The switch off of C/EBPα and the upregulation of MIFT are essential for the commitment of MCp[8].

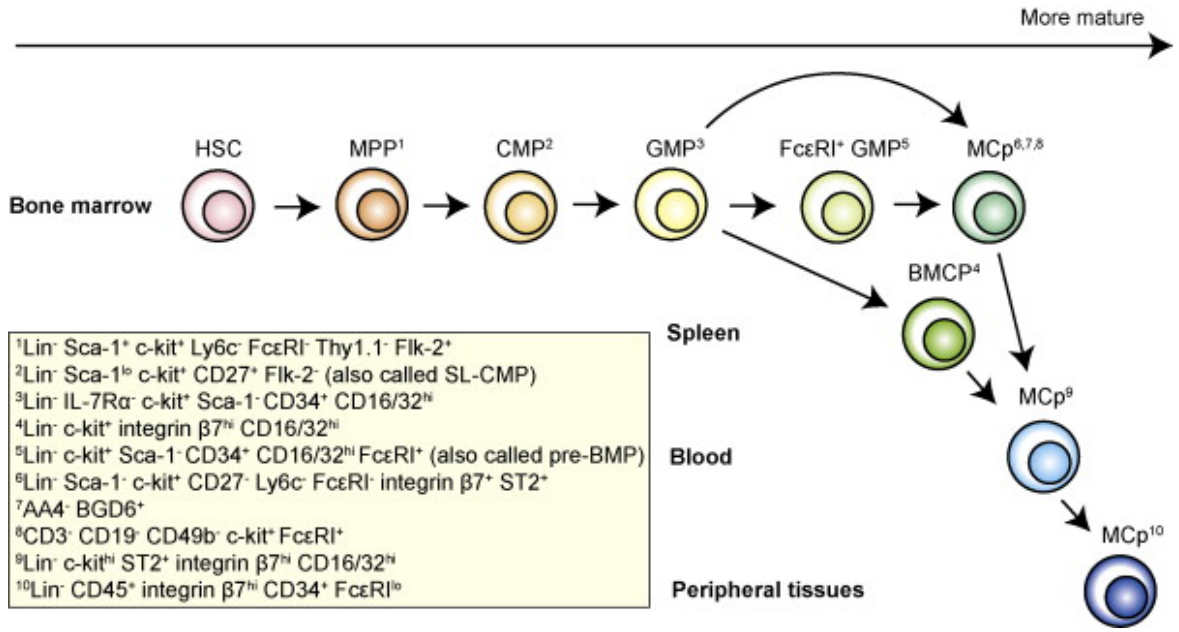


Figure 1: MCp development from the HSC. According to the illustrated model, hematopoietic stem cells (HSCs) originated in the bone marrow differentiate into multipotent progenitors (MPPs), then into common myeloid progenitors (CMPs) and granulocyte/monocyte progenitors (GMPs). The GMPs population give rise to both MCs and basophils not only from an FCεRI+ subset but also from an FCεRI- population. Also MCs' precursors (MCp) can express or not the FCεRI both in the bone marrow and in the blood. In tissues of C57BL/6 mice, MCp express low level of this receptor[8].

In vitro, MCp have a great capacity to undergo cell division[9] but *in situ* proliferation is still a matter of debate. MCs are long-lived cells and, in the periphery precursors acquire, in a microenvironment-driven way, the phenotype of mature cells capable of multiple degranulation during time[10]. MCs are greatly influenced by the modifications of the local environment *(figure 3),* for example, in several diseases, an altered accumulation of MCs is reported. This mastocytosis is likely due to an increased recruitment of immature precursors[8] but mediators such as the stem cell factor (SCF) can also induce the proliferation of on-site MCs during the progression of the pathology[11].

MCs can be found in many different organs[12], with a major representation in the gastrointestinal tract[13], the peritoneum (especially in the murine setting)[14] the skin[15] and the lungs. Rare MCs localized also in organs such as the spleen, lymph nodes (LNs), brain, pancreas and kidney, their numbers can increase with the development of inflammation or cancer[16]. Recently, Gentek et collaborators, with very elegant demonstrations, were able to segregate the hematopoietic

output of the yolk sac and the HSCs produced in the aorta-gonado-mesonephros. They utilized the Cdh5-CreERT2 mice and took advantage of the Cdh5 expression by all endothelial cells and its downregulation upon hematopoietic transition, and applied the temporally defined activation of Cdh5-CreERT2. The authors uncovered that MCs have dual developmental origins in primitive and definitive hematopoiesis: a first wave from the endothelium of the yolk sac that is dilute with a second wave of definitive MCs. Moreover, they observed that adult MC maintenance is generally bone marrow independent, supporting the idea that homeostatic maintenance and replenishment of MCs could be supported by in situ expansion of MCp or mature MCs[17].

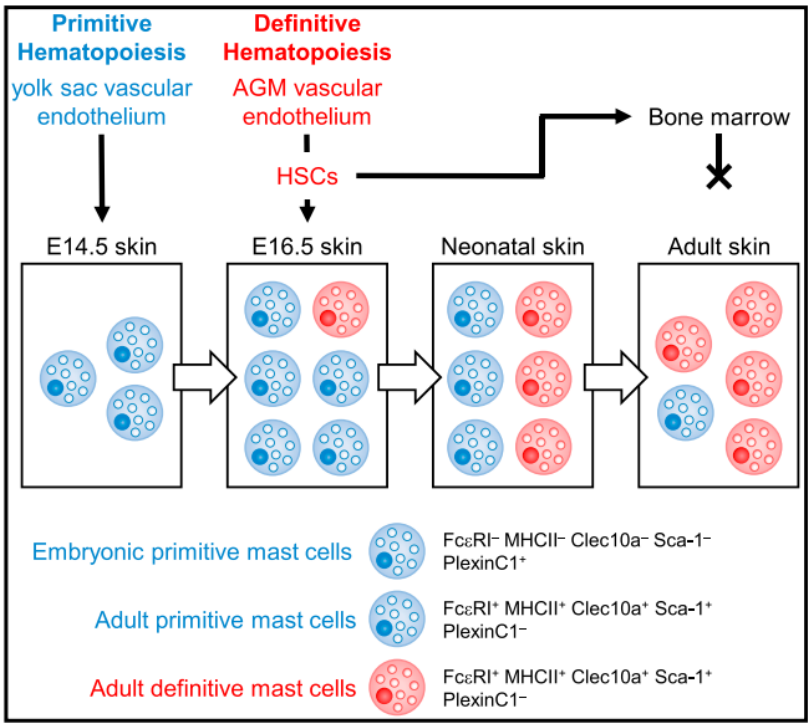


Figure 2: **Model of dual developmental origin of mouse skin MCs**. This pattern shows that MCs can originate not only via primitive haematopoiesis but they are also generated with the definitive adult haematopoiesis. During embryogenesis, skin MCs would derive from the yolk sac vascular endothelium, on the contrary after birth MCs derive from HSCs. Surprisingly, in adult mice MCs homeostasis is independent from the bone marrow and it is suggested that there is a reservoir of tissue-resident MC precursors produced by HSCs during embryogenesis that is able to proliferate. Primitive MCs in adult skin look like adult MCs of definitive origin rather that embryonic primitive MCs (taken from commentary by Anja A. Wolf and Helen S. Goodridg[17]).

The characteristic tissue residency of MCs makes their *in vitro* studies very difficult: processes of direct isolation from tissues or differentiation protocols from MMPs in culture media are required. Murine fetal tissues such as the liver or the spleen are good sources of MCs progenitors. However, the bone marrow of young animals is the most easily accessible source of precursors and allows,

after addition of IL-3 or/and SCF, to induce the differentiation and the maintenance of bone-marrow derived MCs (BMMCs)[18]. Form the peritoneal cavity of rodents, and from a lesser amount the skin, instead, mature connective tissue MCs can be isolated and separated (these cells *ex vivo* are called peritoneal cell-derived mast cells (PDMCs)) [19]. PDMCs need both IL-3 and SCF to survive and amplify *in vitro* after peritoneal cells collection and culture.

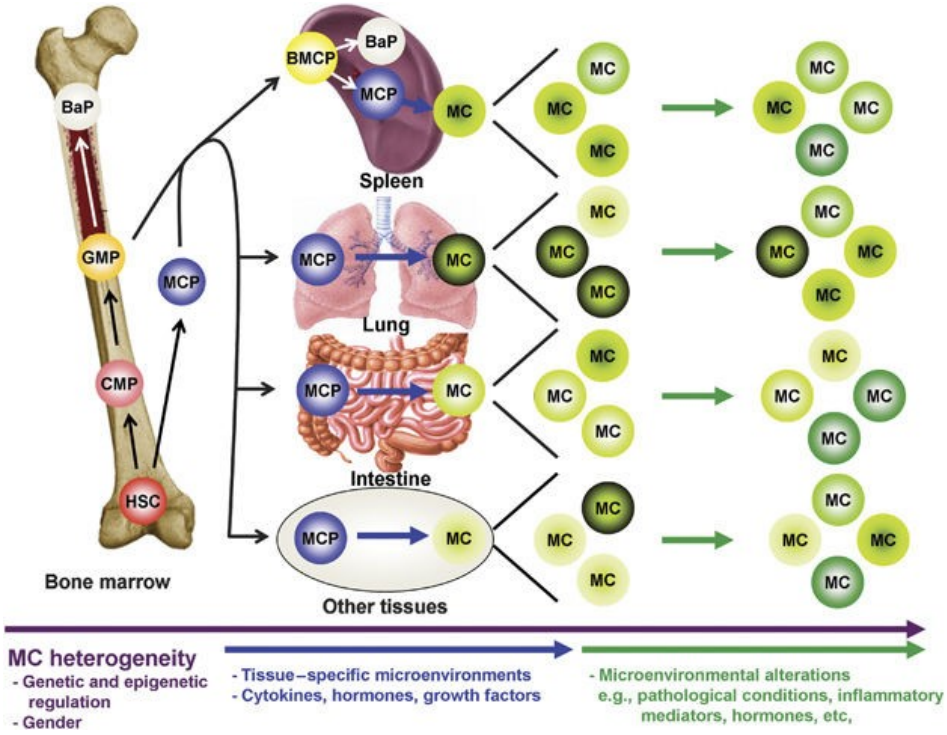


Figure 3: MCp, by effect of different microenvironments, give rise to heterogeneous MCs populations. MCp (MCP in the figure), subject to genetic and epigenetic regulations, originate from the bone marrow and migrate in several tissues. In periphery, they mature under the effect of different microenvironments (cytokines, growth factors, resident cells) acquiring very diverse phenotypes. Moreover, microenvironmental alterations (mediators of inflammation, pathologies etc.) can induce a further change in MCs' phenotype and behaviour[20].

3.1.2. Main categories of MCs' subsets

In mouse mature MCs are subdivided in two main groups based on their anatomical location and granule composition: connective-tissue MCs (CTMCs), mainly localized in serosal cavities, around venules and nerves[21], and mucosal MCs (MMCs). Both categories respond to IgE-mediated activation with signalling, degranulation, and inflammatory cytokines release but differ for biochemical and functional properties. In human the classification is based mostly on protease content. In brief, three main subsets of human MCs can be distinguished: tryptase-positive MCs

(MC_T), chymase-positive MCs (MC_C) and tryptase- and chymase-positive MCs (MC_{CT}). MC_T are more abundant in the alveolar wall and gastric mucosa, resemble MMCs in rodents and are predominantly present in the mucosal tissues of the gut and in the respiratory tract mucosa. MC_C do not have specific localization nor abundancy in tissues, while MC_{CT} are located predominantly in the skin and intestinal submucosa; both are similar to CTMCs in rodents and contain heparin and chondroitin sulphate A and E[22].

Concerning rodents, MMCs differ from the CTMCs for tissue localization and also for morphological and functional properties that are reflected in different histochemical properties and content of granule proteinases (Enerback L., 1987). MMCs express in basal conditions the transcripts for the MC protease-1 (MCPT1) and MCPT2 chymases and contain serotonin but low levels of histamine. CTMCs contain instead both serotonin and high levels of histamine; they express MCPT4 chymase, MCPT5 elastase, MCPT6 and MCPT7 tryptases and carboxypeptidase A3 (CPA3)[23]. The protease content however differs also for anatomical localization and inflammation state: MMCs in the intestine of *T. spiralis* infected mice expresses only MCPT1, while CTMCs expresses both MCPT4, MCPT5, MCPT6, MCPT7 and CPA3[24]. In an allergic inflammation model both tracheal MMC and CTMC expressed all the six proteases, while in the proximal bronchi MMC expressed only MCPT1, MCPT6 and MCPT7 while CTMCs were still positive for all the six proteases[25]. In 2016 Dwyer et collaborators showed that MCs have tissue specific gene expression and among the most differentially expressed genes proteases, G protein-coupled receptors and integrins have been found[26]. To distinguish between MMCs and CTMCs proteoglycan content of the granules are widely used. Two different glycosaminoglycans *(*GAGs) are contained into MCs, heparin and chondroitin sulphates; MMCs contain mainly chondroitin sulphate A, B and E while CTMCs hold both chondroitin sulphate E and high amounts of heparin[27]. The Safranin dye is indeed known to stain with more intensity CTMCs[28].

This heterogeneity can be reproduced also *in vitro*: BMMCs cultured in the presence of the only IL-3 cytokine can be considered as immature MCs with a phenotype that is intermediate between mucosal and connective MCs; indeed they express MCPT5, MCPT6 and CPA3 (which is a CTMC-like phenotype) but the proteoglycan content is more similar to those of MMCs (low levels of histamine and heparin). If BMMCs are instead grown on a monolayer of fibroblast and in presence of IL-3 or the presence of both IL-3 and SCF, the granule content of heparin and histamine are augmented hence these cells become similar to CTMCs. Moreover, the addition of factors such as IL-9, IL-10 or IL-33 induces higher expression of MCPT1, MCPT2 and MCPT6 respectively[29,30].

All these aspects emphasize the great plasticity of MCs, their maturation depends on microenvironmental status and tissue specificity, making reductive the classification based only on granules content.

3.1.3. MC'S biology: activation and mediators

MCs are able to respond to a plethora of different stimuli and the high density of receptors on their membranes makes them particularly sensitive to the microenvironment. Moreover, the great abundance of pre-stored and newly synthesized mediators makes these cells also powerful modulators of the tissue microenvironment. MCs recognise pathogens through toll-like receptors (TLRs), C-type lectin receptors (CLRs), complement receptors and respond to soluble mediators released in the context of infection through several cytokine- and chemokine-receptors[31]. MCs degranulation can occur in response to various external stimuli among which IgE receptor cross-linking is the most known, but they can also rapidly release their mediators in response to complement activation, neuropeptides and certain toxins. Depending on the type of the stimulus they can be activated by the binding of the IgE to the FcεRI in the cross-linking of bi- or multivalent antigens (Ags), or by the binding of the FcγRs by immune complexes (this last receptor in human MCs); but their activation can also be inhibited by the binding of receptors such as the FcγRIIb[32].

3.1.3.1. FcεRI dependent activation

This type of MC's activation is the best characterized and the most studied since, traditionally, MCs have been mostly considered as essential components of IgE-mediated class type I hypersensitivity reactions. The signal begins with the cross-linking of IgE/Ag complexes on MCs' surface FcεRI receptor. This is a tetrameric receptor which contains the IgE-binding *a*-chain, a membrane-tetra spanning *b*-chain and a disulphite-linked homodimer of the *g*-chain. The Ag is recognized by the FcεRIα-bound IgE that induces the receptor aggregation, the activation of SRC family kinases (SFKs) and tyrosine phosphorylation of the receptor subunits. Four main SRC kinases have been described in FcεRI activation: LYN, SYK, FYN and HRC. These SFKs simultaneously initiate independent cascades that induce a complex intracellular signalling that controls MC's activation. This process is tightly regulated: two main complementary pathways are described, the first comprises LYN and SYK and activates the phospholipase Cγ (PLCγ), the second one depends on FYN and activates the

phosphatidylinositol 3-kinase (PI3K)[33]. All these events are key factors during allergic inflammatory responses and the two pathways are briefly illustrated in *figure 4.*

The canonical signalling of the FcεRI

The highest activation is guaranteed by the presence of LYN kinase in lipid raft domains at the plasma membrane. LYN is responsible for the phosphorylation of tyrosine residues in the FcεRI β-chain and γ-chain that are present in the immune receptor tyrosine-based activation motifs (ITAMs). When phosphorylated, the β- and γ-chain ITAMs provide high-affinity docking sites for the SH2 domains of LYN22 and for the SH2 domains of the ZAP70 (ζ-chain-associated protein kinase of 70 kDa)- related tyrosine kinase SYK (spleen tyrosine kinase), respectively. This allows trans- and autophosphorylation of its catalytic domain, as well as phosphorylation by LYN, hence increasing its catalytic activity[34]. SYK and LYN promote the tyrosine phosphorylation of the transmembrane adaptor molecule LAT that is fundamental for the initiation of the downstream signalling pathways and serve as scaffold for recruitment of other proteins[35]. Its four terminal tyrosine residues (Y132, Y171,Y191 and Y226) are reported to be crucial and sufficient for the ability of LAT to regulate degranulation signalling in MCs[36]. The most important protein that is recruited by LAT, is PLCγ which catalyses the hydrolysis of phosphatidylinositol-4,5- bisphosphate (PtdIns(4,5)P2) in the plasma membrane inducing the formation of two products inositol-1,4,5-trisphosphate (InsP3) and diacylglycerol (DAG). The InsP3 induces a transient but sustained mobilization of cytosolic calcium and DAG induces the activation of protein kinase C (PKC). These two signals are essential for the induction of the degranulation[33]. In the final steps of this process, the guanine-nucleotide-exchange factors VAV56 and SOS57 are needed to induce cytokine production: they induce the shift to the GTP bound-active state of RAS. RAS in turns regulates the RAF-dependent pathway that leads to phosphorylation and activation of the mitogen-activated protein kinases (MAPKs) extracellular-signal-regulated kinase 1 (ERK1) and ERK2, JUN amino terminal kinase (JNK) and p38[37]. Finally, together with the calcium influx and the subsequent nuclear translocation of nuclear factor of activated T cells (NFAT), these pathways lead to the activation of NF-*k*B, the activator protein 1 (AP1) members JUN and FOS and therefore cytokine expression is induced[38].

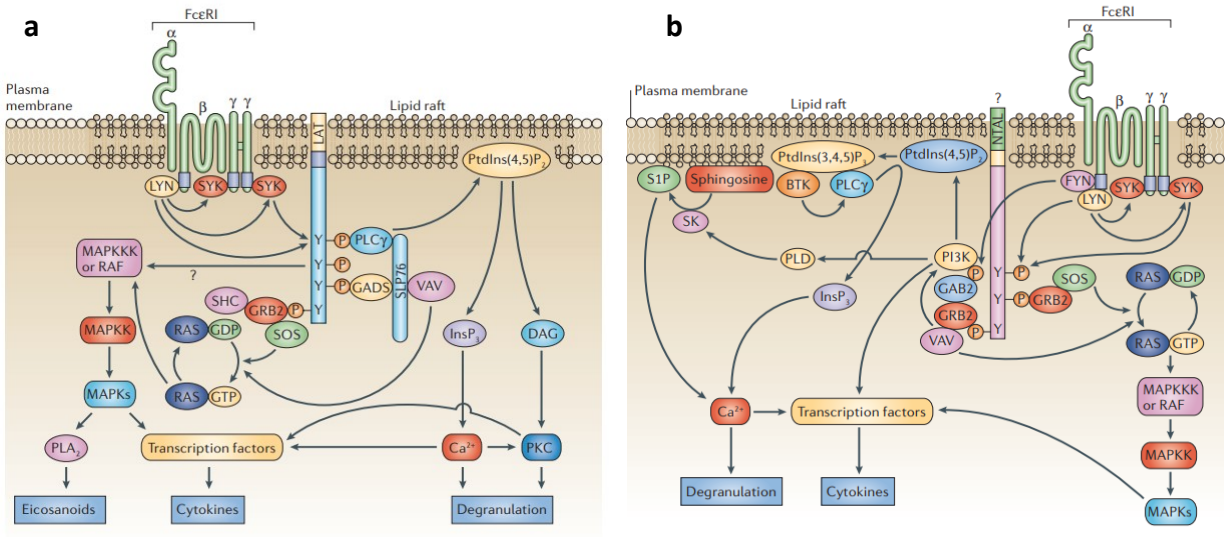


Figure 4: Main and complementary signalling from the FcεRI in MCs: (a) The canonical FcεRI pathway includes a series of consecutive stages from the FcεRI aggregation starting from LYN that associates with ITAM domains and induces SYK. Finally LAT is phosphorylated and it accommodates the signalling required for the production of the different pro-inflammatory mediators: the activation of PLCγ and PKC and the increase of calcium mobilization are essential for the degranulation, at the other side the mitogen MAPK pathway is fundamental for cytokines production. **(b)** In the alternative pathway FYN- and GAB2-dependent phosphorylation of PI3K catalyses a further calcium influx which sustains the initial PLCγ-dependent calcium mobilization and the activation of the AKT/mTOR pathway. NTAL might regulate these processes by binding GRB2 following phosphorylation of NTAL in a LYN- and SYK-dependent manner. Adapted from[33].

The complementary activation pathway of the FcεRI

The discovery of the IgE/Ag complementary activatory pathway arose with the discovery that in addition to LYN, FYN, another SRC-kinase, was essential for degranulation and cytokine production in MCs[39]. LAT, in this pathway, is not essential and PLCγ, on the contrary, is not activated. PI3K is activated through the phosphorylation of the cytosolic adaptor molecule GAB2 (GRB2-associated binding protein 2)[40]. In this process PI3K phosphorylates membrane-bound PIP2 to phosphatidylinositol 3,4,5-triphosphate (PIP3) that works as a docking site for other molecules such as PLCγ, VAV, the Burton's tyrosine kinase (BTK) and AKT. PLCγ phosphorylation induces the calcium influx and PKC activation, AKT is instead fundamental for the activation of the mammalian target of rapamycin (mTOR) pathway by inactivating its negative regulator mTOR tuberin[41]. Active mTOR forms two complexes, the rapamycin-sensitive mTOR complex 1 (mTORC1) and the rapamycin-insensitive mTORC2.

Animal studies showed that PI3K seems to be mainly responsible for the maintenance, but not for the initiation, of the calcium signal that is required for an optimal degranulation. It is not well

defined whether for calcium mobilization and degranulation, an adaptor transmembrane molecule is required, it has however been proposed that NTAL, known also as LAT2, can have similar functions covered by LAT in the assembly of the macromolecular complex necessary for FYN signalling[42].

3.1.3.2. Other ways of activations

As mentioned above, the IgE/Ag-dependent activation of MCs is the best known activation process but cannot be considered the most important since in different types of inflammatory diseases or tumors, where MCs' accumulation has been proposed as a prognostic role, they are reported to be activated independently from an IgE stimulation[43,44].

Different products of pathogens, such as the lipopolysaccharide (LPS), the peptidoglycan (PGN), poly (I:C) and some viruses are able to directly interact with MCs through the TLRs expressed on their membranes: TLR-1, 2, and 4–6 on the cell surface while TLR-3, and 7–9 intracellularly[45]. TLR signalling includes a complex network of signalling molecules and, among others, the MyD88-dependent and MyD88-independent pathways are highly relevant. The type of cytokine produced after a TLR activation in some cases is redundant, nevertheless some differences can be found, TLR-2 activation induces the production of TNF-α, IL-6, IL-13, IL-4, and IL-5, while TLR-4 leads to the synthesis of TNF-α, IL-6, IL-13, and IL-1β. One interesting aspect is that if murine MCs are pre-exposed to TLR ligands, the following IgE-induced activation is suppressed because of the temporary reduced FcεRI expression[46]. Oppositely, in human MCs it has been shown that a simultaneous exposure to various TLR ligands and FcεRI stimulation produces an increased cytokine secretion without affecting degranulation[47].

MCs can also be activated by IgG immune complexes binding pro-inflammatory FcγRI, FcγRIIA, or FcγRIII and many works have reported a role for FcγR-induced mast cell activation in pathologies like rheumatoid arthritis (RA), Sjögren's syndrome, multiple sclerosis, or glomerulonephritis[48].

Moreover, degranulation is induced also by activation of the complement fragments C3a and C5a that are also called anaphylatoxins. C3a sustains MCs' degranulation in the presence of FcγRI signalling and C5a is able to induce migration and adhesion aside from cytokine synthesis[49,50].

On their surface MCs present a wide range of cytokine and chemokine receptors. Chemokines are important in directing MCs to the anatomical compartment where they will accomplish their differentiation, in inflammatory conditions their receptors respond to chemokine gradients

favouring a specific and localized activation. Chemokine receptors that are constitutively expressed in MCs include CCR1, CCR3,4 and 5, CXCR1–4, and CX3CR1[51].

Cytokines instead are essential meditators that control many homeostatic functions such as growth and proliferation but also promote activation or silencing of specific signalling. Among the ones that drive homeostatic activities, and besides the already cited importance of IL-3 and SCF that activate several antiapoptotic pathways, IL-4, IL-10, and TGF-β are very relevant[52].

The IL-33/ST2 axis in MCs

IL-33 cytokine is acquiring more and more importance in MC biology since it acts as a potent activator. ST2 and IL-1RAcP on MCs form a transmembrane protein complex that is the only known receptor for IL-33. This interleukin triggers a MyD88-dependent NF-kB signal similar to the TLR cascade and promote survival, maturation, cytokine production and adhesion. More specifically, IL-33 binding to ST2 leads to the recruitment of MyD88 adapter protein along with IL-1R-associated kinase1 (IRAK1), IRAK4 and TNFR-associated factor 6 (TRAF6)[53] then NF-κB and MAP kinases transcription factors become activated; as well as ERK1/2, p38MAPK, and JNK kinases are activated and inflammatory mediators are produced[54]. The first report about cytokine polarization concerning an IL-33 stimulation of MCs concern a Th2-cytokine polarization[54], however the impact of IL-33 on mast cell biology has also been extended at the single cell level. It has been demonstrated that IL-33 potentiates most of the IgE-mediated MC responses both by increasing the number of responding cells and by enhancing the responses of individual MCs[55]. Additionally, it enhances cytokine production amplifying the IgG signals[56]. In *table 1* are listed the main effector mediators induced after ST2-dependent signalling.

Table1: Functional effects of IL-33 on MCs. The principal effects of the downstream signalling cascade of IL-33 binding on ST2 on BMMCs, human LAD2 cell line, human umbilical cord blood-derived MCs and murine intestine and lungs. MCs upon effect of IL-33 are supported in their survival, cytokine and chemokine production.

Cytokine	Downstream signaling cascade	Cell type	Functional effect
IL-33	MyD88	BMMCs	Survival of BMMCs
	MyD88	BMMCs	Production of cytokine e.g. IL-6 and IL-13
	MyD88	BMMCs	Proliferation of mast cell
	MyD88	BMMCs	Release of IL-6 and IL-13
	MyD88	Intestine (mice)	Production of type 2 cytokine e.g. IL-4, IL-5 and IL-13
	MyD88	Lungs (mice)	Goblet cell hyperplasia
	p38 MAPK	BMMCs	Proliferation of mast cell
	p38 MAPK	BMMCs	IL-6 release
	p38 MAPK	BMMCs	IL-6 and IL-13 release
	JNK, ERK, p38 MAPK, NFκB	BMMCs	IL-6 and IL-13 release
	NF-κB and JNK1/2, ERK1/2, and p38 MAPK	BMMCs	Production of IL-4, IL-5, IL-13, CCL2, CCL17, and CCL24
	p38 MAPK	Human mast cell LAD2	IL-13 release
	p38MAPK	Human umbilical cord blood-derived mast cells (HUCBMCs)	IL-8 release

Historically MCs were among the first cell types to be identified to express the ST2 receptor. IL-33/ST2 activation of MCs is growing in importance since more and more implications of this axis in pathological conditions such as asthma, anaphylaxis, arthritis, or tumours are discovered[57,58,59,60]. An important aspect to note is that MCs are not only activated by IL-33, they are also among the cells that are able to actually produce the cytokine. IL-33, that is stored in the nucleus, is also considered an “alarmin” since it is released after necrosis or tissue damage, instead when apoptosis occurs it is inactivated by caspases cleavage. It is mainly expressed by structural cells as oblasts, osteoblasts, endothelial cells, epithelial cells but also in MCs, dendritic cells (DCs) and macrophages[61]. It is furthermore reported that chymase and tryptase, the two major serine proteases released by activated human MCs, are involved in the processing of IL-33. The mature forms of IL-33 generated resulted to be potent activators on type 2 innate lymphoid cells (ILC2s), and this suggests that both MMCs and CTMCs in their different microenvironment, might be able to generate highly active mature forms of IL-33[62].

The MC’s involvement in the production of IL-33 in *in vivo* pathological setting has been inquired. The first findings demonstrated a mechanism by which MCs influence the recruitment and activation of inflammatory cells into sites of IgE-dependent activation pointing out a functional effect of endogenous IL-33 in inflammation. Nevertheless, deciphering the mechanisms through which IL-33 becomes extracellular is still an unexplored area of study, indeed experiments have failed to detect IL-33 release from MCs *in vitro*[63]. It has been suggested that failure to detect IL-33 release *in vitro* lies in the limitations of the existing cytokine detection techniques enzyme-linked

immunosorbent assay (ELISA), and a modified method to allow detection of IL-33 from the cell supernatant has been suggested[64].

3.1.3.3. MC's derived mediators

The most common identified morphological feature of MCs is their high cytoplasmic content of electron-dense lysosome-like secretory granules. Indeed a great abundance of pro-inflammatory mediators is stored and released from these cells when appropriately activated. For this reason MCs have been defined as "cells armed for a battle"[65].

In response to some stimuli, beyond the classical degranulation process, MCs also display a "piecemeal" degranulation characterized by gradual loss of granule contents without detectable granule fusion to the membrane. Thus, piecemeal degranulation allows release of discrete packets of granule-associated components without granule exocytosis[66]. Mediators released from MCs are divided into two groups: pre-formed mediators, such as histamine, that are stored in the cytoplasm in granules, and *de novo* synthesized mediators that comprise lipid mediators, cytokines, and chemokines. Very different roles are played by this bioactive molecules, not only in allergic settings, but also in physiological and pathophysiological conditions[67]. In the granules vaso- and neuro-active molecules such as histamine, serotonin, β-exosaminidase, tryptase, chymase are stocked. Growth factor such as epidermal growth factor (EGF), fibroblastic growth factor 2 (FGF2), granulocyte-macrophage colony stimulating factor (GM-CSF), transforming growth factor type-β (TGF- β), vascular endothelial growth factor (VEGF) and lipid mediators, including leukotriene (LT) C_4, prostaglandin (PG) D_2, and platelet activating factor (PAF) are neo-synthesized and are good example of how diverse is the MCs contribution in modulating biological processes[68]. A deeper description of MCs' derived cytokine, bioactive molecule that are mainly involved in the regulation of other immune cell types is done below.

TNF-α (tumor necrosis factor-alpha)

This mediator has been historically discovered to be expressed in MC lines (e.g. C57.1, 2D4, RBL-2H3), IL-3 maintained BMMCs, rat and mouse peritoneal MCs (PMCs), rat CTMCs and also human bone marrow-derived "basophils/MCs" and PMCs (stored preformed)[69]. The mRNA expression and production of this mediator has been shown to increase upon an IgE-dependent stimulation[70].

Moreover IL-33 together with substance P (SP) has been shown to enhance the TNF-α synthesis and secretion in LAD2 human mast cell line[71]. In addition, IL-33, through the signalling induced by the binding of its receptor ST2, is reported to be critical for the late-phase inflammation occurring during FcεRI anaphylactic activation of BMMCs[63].

The evidence of preformed TNF-α in MCs has been demonstrated in MC-deficient *Kit*$^{W/W\text{-}v}$ mice where rapid secreted TNF-α helped the initiation of local inflammation by recruiting neutrophils into the peritoneal cavity[72].

As mentioned above, one of the first roles uncovered for MC-derived TNF-α is the recruitment of other leukocytes during an inflammatory process, not only neutrophils during hypersensitivity reactions[73], but also the migration of skin or airway DCs[74]. Also adaptive immune cells can be affected by IgE-activated MCs through the release of TNF-α, as reported in a work in which T cells activation was increased through the expression of OX40[75]. It is important to note that not only immune cells respond to the TNF-α molecule, keratinocytes for instance are induced to produce nerve growth factor in response to MC-released TNF-α that in turns induce nerve elongation[76].

The results section of this thesis will show a novel and important role that MCs-derived TNF has in a context of intestinal tumorigenesis.

Other pro-inflammatory cytokines

IL-6 is a pro-inflammatory cytokine that is produces by many immune cells and well-studied in MCs because is largely released from this cell type. MCs are able to produce IL-6 in response to an IgE-dependent stimulation, a TLR4-dependent activation, after SP, IL-1 or IL-33 encounter. In addition, IL-6 can act also in an autocrine way since human MCs growth is supported by its anti-suppressive signalling[77]. Similar stimulations are needed for the release of IL-1β that is shown to be implicated in arthritis or skin inflammation[57]. MC-derived IL-6, together with INF-γ, a Th1 cytokine, are shown to be relevant in promoting atherogenesis[78].

Among the Th2-type cytokines, IL-4, IL-5 and IL-13 are the most counted and their functions partially overlap. These cytokines are released upon an FcεRI stimulation or under LPS, IL-33, PGN stimuli. IL-13 plays an important role in host defence to infection against parasites and together with the other cytokines participates in the pathogenesis of type-2 immune responses that are usually associated with high antibody (Ab) responses[79,80].

IL-10 is an anti-inflammatory cytokine secreted mostly by regulatory T and B lymphocytes, macrophages, DCs, Natural Killer (NK) lymphocytes and others. MCs release and production of IL-10 is increased by LPS, IgE-crosslinking and via FcγRIII[81]. Reports indicate a role of MC-derived IL-10 in the suppression of adaptive responses ameliorating the detrimental effect contact hypersensitivity, this can also have negative consequences by driving tolerance in a model of bladder *E. coli* infection[82,83].

Chemokines

Chemokines are a particular class of cytokines with a generally low molecular weight, ranging from 7 to 15kDa and a chemotactic activity on immune cells. Chemotaxis is a key process in homeostasis, it allows migration and compartmentalization of cells in specific body sites, and is also extremely relevant during the onset of inflammatory processes including infections, allergy, autoimmune diseases and tumors[84,85]. For this reason one type of classification is the division into homeostatic and inflammatory chemokines. Moreover two main families of chemokines can be identified based upon cysteine residues position: CXC and CC (even though historically they have been known with different names). In 1888 Leber was the first to describe chemotaxis reporting the movement of leukocytes towards inflammation sites. This phenomenon is induced by the formation of an extracellular gradient of chemicals. Cells that express on the membrane the corresponding chemokine receptor (or more than one receptor), become polarized and migrate towards the higher chemoattractant concentration. Chemokine receptors are differentially expressed by immune cells and can be divided into two groups: G protein-coupled chemokine receptors (GPCRs) and atypical chemokine receptors, which seem to form chemokine gradients and reduce inflammation by scavenging chemokines in a G protein independent manner[86].

It is known indeed that among the newly synthesised mediators, MCs are source of a wide plethora of chemokines. Some of them are expressed both in mouse and human such as CCL1, CCL2, CCL3, CCL7, CCL17, CXCL4, CXCL5, some have been detected only in mouse studies such as CCL21 and CCL25, and others are described only in human MCs: CCL4, CCL5, CCL19, CCL20, CXCL10[87].

In this work, chemokines and the ability of MCs in regulating chemotactic properties of B cells in physiology and during cancer development is of great interest.

3.1.4. Animal models for studying MCs *in vivo*

To date, Abs-based methods or stabilizers able to specifically inhibit MCs activity are not available, therefore genetically modified animals are the most used and suitable. Indeed, over the past decades many efforts have been put in generating MCs-deficient animal models to study specific functions of MCs. However, each approach has revealed limitations that need to be taken into account in the interpretation of the results.

The first MCs deficient mice harboured naturally occurring a loss-of-function mutations at the Kit-encoding locus (also known as white spotting locus W). Differently from the majority of the hematopoietic cells that lose Kit expression upon differentiation, MCs express Kit all over their lifetime and binding the SCF participate in the survival, differentiation, self-renewal, proliferation and migration[88]. In the *in vivo* studies, *WBB6F1-Kit$^{W/W-v}$* and *C57BL/6-Kit$^{W-sh/W-sh}$* mice are the most used Kit mutant mice.

The first mice carry one allele (W) with a point mutation which encodes a truncated version of Kit, and one allele (W-v) with a point mutation in the tyrosine kinase domain of Kit. The second model, **C57BL/6-Kit$^{W-sh/W-sh}$**, instead presents an abnormal Kit expression due to an inversion mutation that affects the transcriptional regulatory elements upstream of the c-kit transcription site on chromosome 5. As a result, in these mice all MCs subsets are depleted. Unfortunately, the loss of Kit causes other major defects: both mice are greatly deficient in melanocytes, conferring the white coloured fur. In addition, WBB6F1-Kit$^{W/W-v}$ mice are anaemic and sterile and have reduced numbers of neutrophils and basophils; C57BL/6- Kit$^{W-sh/W-sh}$ mice instead are not anaemic nor sterile, but have increased numbers of neutrophils and basophils and exhibit splenomegaly[89].

From the work of Nakano T. and collaborators in 1985, many anatomical sites of these Kit-mutant mice are engrafted with genetically compatible *in vitro*-derived Wt or mutant MCs (such as BMMCs). This allows to attribute to MCs specific effects observed in these mice that carry strong non-MCs related abnormalities. The principal routes for the administration of MCs, in order to generate this sort of “MC knock-in mice”, are intraperitoneal (i.p.), intravenous (i.v.) and intradermal (i.d.). However it has been demonstrated that upon reconstitution different body compartments are differently engrafted with MCs compared to normal tissue distribution. For example, using i.p or i.v. reconstitutions native levels of skin MCs are not restored; on the other side, an i.v. injection is not able to refurbished peritoneal cavity MCs number as well as in the spleen, an organ in which the

density of MCs is particularly low, collectively, this type of reconstitution leads to a non-physiological accumulation of MCs[90].

More recently, new strains of MC-deficient lacking abnormalities related to c-kit structure or expression mice have been created. The most common transgenic mice carry Cre recombinase (Cre) expressed under the control of MC-specific or MC-associated promoters. Mcpt5 gene is the target gene of the *Mcpt5-Cre;R-DTA mice.* To generate these mice Cre has been crossed with Mcpt5 and Cre-specific ablation is specific in cells expressing the diphtheria toxin alpha (DTA)[91]. There is a constitutive lack of peritoneal and ear skin MCs, an almost complete reduction in the number of abdominal and back skin MCs, but numbers of MMCs that do not express Mcpt5 are not affected.

In the *Cpa3^{Cre}/+ - "Cre-Master" mice*[92] the mice are extremely depleted of MCs and this seem to be due to Cre-induced genotoxicity. A limit of this model is the reduction of some other populations that express various levels of Cre such as other hematopoietic progenitors, basophils and tymic T cells[89].

A third mouse model, derived from the previous one is the *Cpa3-Cre;Mcl-1$^{fl/fl}$* called *"Hello* Kit*ty" mice* since the phenotype of MCs deficiency occurs in the absence of mutations of the c-Kit. These mice, in addition to have almost 100% of MCs depletion in all anatomical sites, have a great reduction in basophils numbers[93].

The principal goal in studying MC-deficient animal models is to validate the role and the contribution of MCs in certain pathologies and to assess them as therapeutic targets. The ideal *in vivo* model should be the one in which MCs depletion is both highly selective and inducible. Current promising approaches, make use of the injection of diphtheria toxin (DT) into transgenic bearing DT receptor (DTR) adult mice into selected cell types.

Below the ***B6; B6.Ms4a2tm1Mal*** also called **RMB (Red Mast Cell and Basophil) mice** is described; this model was generated by the group of Pierre Launay in 2014[94] and is also used in the present thesis work. In this mouse model the 3'-UTR of the FcεRI β-chain, encoded by the Ms4a2 gene, holds a cassette composed of an internal ribosomal entry site, a sequence coding for the bright red td-Tomato (tdT) fluorescent protein, a 2A cleavage sequence, and the human diphtheria toxin receptor (hDTR). This construct allows the tracking of both MCs and basophils thanks to the red fluorescence and makes these cells sensitive to DT. In *figure 5* the whole strategy used to create the RMB mice is shown. Two i.p. injections of DT (within 48h) are needed to obtain a complete depletion of MCs in the peritoneal cavity and other compartments, and of basophils in the blood. The analysis of the repopulation kinetics demonstrated the adequacy of the model: basophils started to reappear 6

days after the DT treatment and reached their normal levels by day 12. Differently, MCs were not detected within 12 days nor 2 months after DT treatment the numbers of MCs remained low (about 6% of the physiological conditions); their half repopulation was seen at 6 months after the depletion.

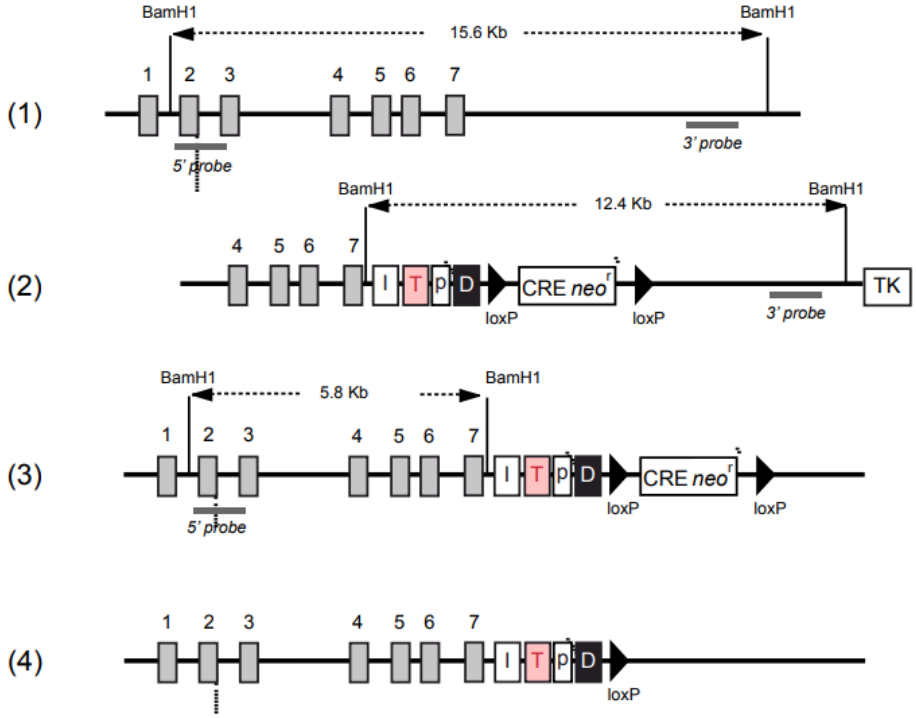


Figure 5: Strategy used for the generation of RMB knock-in mice. (1) The Wt Ms4a2 gene that encodes for the FcERI β-chain has been partially restricted. Exons are represented as grey boxes. Two BamH1 sites are used for digestion and the 5' and 3'of single-copy probes used to verify proper homologous recombination are shown. (2) An IRES-tdTomato-2A-hDTR cassette in the 3' untranslated region of the gene is introduced: this cassette is flanked at its 3' end by an auto-deleting loxP-Cre-NeoR-loxP cassette. TK: thymidine kinase expression cassette bordered onto the 3' end of the right homology arm. I: IRES, T: tdTomato, p: peptide 2A, D: hDTR. (3) This is how the structure of the targeted allele following homologous recombination and preceding Cre-mediated self-excision of the neoR cassette appears. (4) Final structure of the target site after auto-deletion of the Cre-NeoR cassette in male gametes.[94]

By investigating the RMB derived BMMCs' properties, the authors determined that B6 BMMCs characteristics where comparable with them. Moreover, the numbers of other leukocyte populations (neutrophils, eosinophils, monocytes, B and T lymphocytes) were not affected in this knock-in RMB mouse proposing this as an ideal model to study the homeostatic and pathological role of MCs. In their work, Dahdah A. and collaborators were able to prove a detrimental role for peritoneal MCs in a model of severe sepsis since IL-4 produced by this MC subset by effect of *E. coli* was responsible for inhibiting resident macrophages phagocytosis. This model can be therefore considered better compared to Kit mutant mice (even if different, since these mice are MC-competent until their induced depletion), it is however important to keep in mind that in humans

there is no report of any MCs deficiency and it is likely to consider that any lack of MCs does not provoke severe immunodeficiency[95].

3.1.5. The MC as an highly social immune cell

MCs are known to give a high contribution in the regulation of innate and adaptive immune responses. They can be considered highly promiscuous cells, indeed their broad array of cell surface receptors, co-stimulatory molecules, and ligands involved in cell–cell contact (and also in cell-extracellular-matrix adhesion) make them important modulators of other immune and non-immune cell types, reflecting in very complex bi-directional interactions[87]. We can consider MCs as highly specialized cells in creating communication with other cells and many reports have shown the clinical relevancy that this modulation of other cell responses produce in the overall improvement of the disease[96] .

Eosinophils are closely related to MCs since both of them are key effectors in allergies, in this light their interaction is important in the frame of the regulation of allergic responses. Human cord blood derived MCs and human peripheral blood eosinophils are able to mutually increase the survival and activation *ex vivo* in the presence of SCF[97].

MCs and basophils cooperate in worsening or modulating inflammation as well as in facilitating tissue repair. Human basophils chemotaxis is induced by the binding of PDG2 released by MCs to the transmembrane receptor CRTH2, expressed on basophils[98]. During lung inflammation, CCL2 secreted by MCs can induce histamine and leukotrienes release from human basophils[99]. MC-derived VEGF concurs with basophil recruitment at inflammatory sites and then promoting angiogenesis. Moreover basophils can be activated by a wide plethora of receptors for growth factors released by MCs such as IL-2, IL-3, IL-4, IL-5, GM-CSF, NGF and IL-8[100].

The involvement of MCs in the activation of neutrophils became clear with the first study of reconstitution of MC-deficient mice[87]. In a model of immune complex-mediated peritonitis both the rapid and the late phase recruitment of neutrophils appeared to be promoted respectively by LTs and TNF-α produced by MCs[72], also CXCL2 and mMCP-6 cover a role in neutrophils chemotaxis induced by MCs facilitating host defence[101,102]. Recently a close morphological association between MCs and neutrophils was documented in a series of human gastric cancer. The ultrastructural analysis allowed to uncover degranulating MCs with a mechanism called *kiss-and-run* fusion that is different from the classical massive anaphylaxis or the piecemeal degranulation[103].

In the modulation of innate immune responses, MCs release factors that are able to affect DCs such as histamine, prostaglandins and TNF-α that are known to facilitate their migration, maturation and antigen processing [74,104,105]. More recently it has been shown that, beyond soluble mediators, also the cell-cell contact is established between these two cells, a synapse allows the transfer of antigens that in turns leads to T cell activation[106]. In addition, the formation of direct contact between these two cell type depends on, the axis between lymphocyte function-associated antigen (LFA-1) on DCs and intracellular adhesion molecule (ICAM-1) on MCs [107].

The interactions with monocytes and macrophages are extensively studied in the context of infectious conditions, however monocytes and MCs have been shown to accumulate also in several inflammatory states, for example interaction of the two cells have been proposed in cardiovascular and metabolic diseases[108]. The Th2 cytokine IL-4 is one of the reported factors derived from MCs that are able to affect macrophage activity in the context of infections. However opposite roles have been shown: promotion of an efficient phagosomal activity by macrophages[109], and downregulation in a septic model instead[94].

MCs are not only modulators of innate immunity, many studies indeed show their ability to finely tune the activities of effector T, B and regulatory lymphocytes bridging innate with adaptive immunity. MCs-B cells interplay is of main interest for the purpose of this work and is extensively discussed in the *section 3.3.1 "MC-B cell cross-talk: what is known and what is missing"*.

In T cell-mediated inflammatory processes, a physical proximity and an increased number of MCs are described. Recruitment, activation, proliferation and cytokine production are modulated by effect of MCs in several T cell subsets[110]. MCs expressing CD80/CD86, PD-L1, ICOS-L, OX40L co-stimulatory molecules, together with the induction of MHC-II expression (primed by a combination of inflammatory stimuli such as INF-γ, LPS and IL-4) are able to act as antigen presenting cells[75]. It is interesting to note that a role for MCs-derived exosomes are able to induce antigen specific responses of T cells[111]. The sustained T cells' activation and cytokine synthesis and a polarization towards a Th2 $CD4^+$ cells is induced by TNF-α and by other important mediators (IL-4, IL-6, IL-13) release by MCs[112]. Interesting positive and negative activation of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg) roles are played by MCs. MC-derived IL-6 and interactions through OX40 on T cells and MC-expressing OX40L have suppressive role in Treg functions[113]; also histamine, released during a microbial activation of MCs has been proposed to have a negative effect of Treg activity[114]. There are reports in which a greater MCs recruitment is observed into the peritoneum in mice with Treg

transfer suggesting a MC contribute in Treg activity[115]. Moreover, Treg, differently from effector T cells, inhibit MC activation and release of mediators (illustrated in *figure 6*).

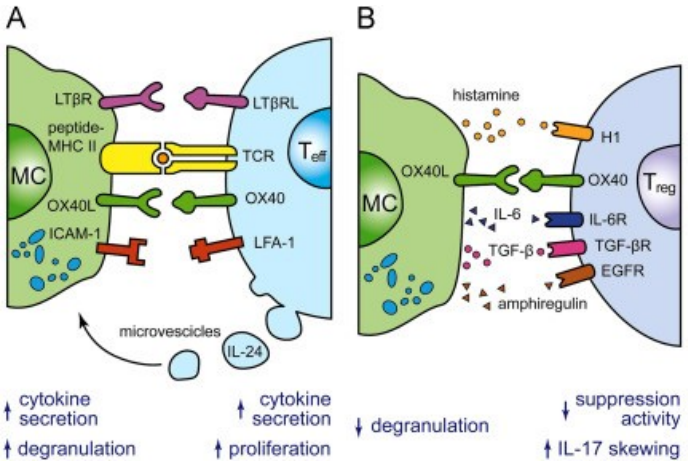


Figure 6: Molecules and soluble mediators involved in MC-T cell crosstalk: (A) Effector T cells are shown in their crosstalk with MCs. Different membrane axis promote a mutual sustain in cytokine secretions and activation of the cells. **(B)** MCs interaction with Treg downmodulates MC degranulation. Modified from[113].

3.2. THE B CELL ARM OF THE IMMUNE SYSTEM

3.2.1. B cell development: B-1 and B-2 cells the main subsets

B cells are an important component of the adaptive immune system. Differently from the cells of innate immunity, defence against pathogens is achieved by a mature compartment of long lived lymphocytes that underwent differentiation capable of providing Ag presentation and production of Abs. This mature compartment, made of different subtypes of B cells with complementary functions, is supplied by a continuous production of immature cells from the bone marrow. In mouse, two main categories of B cells can be identified, B-1 and B-2 lineages that are distinguished by their developmental origin, anatomical localization and functional properties[116]. In general, B-2 cells are "conventional" B lymphocytes, that participate in adaptive immune response undergoing germinal centre (GC) reactions, mainly in the spleen and LNs. After migration in the spleen, B-2 cells can further mature into follicular (FO) or marginal zone (MZ) B cells[117]. B-1 cells instead can be considered as "innate" B lymphocytes that reside mainly in the body cavities. The origin of the B-1 and B-2 main subsets has been long debated, but the majority of the studies suggests the existence of different progenitors[118].

3.2.1.1. B-1 B cells

Historically this murine B cell subset has been identified as $CD5^+$ B cells sharing similarities with human chronic lymphocytic B-cell leukemia[119]. Later B-1 cells were identified as the dominant B cell population in the peritoneal and pleural cavities and extremely rare cells in spleen or LNs. Phenotypically they express high levels of surface IgM, low levels of B220 and IgD and are $CD23^-$ cells. Moreover, B-1 cells can be further divided on the base of CD5 expression into B-1a ($CD5^+$) and B-1b ($CD5^-$). B-1 cells residing in the peritoneal cavity express in addition intermediate levels of Mac-1. B-1 cells express at high levels different type of integrins (α4, α6, β1 and β7) and the CD9 tetraspanin[120]. In addition, in the body cavities, they are defined as cells having a $CD11b^+sIgM^{hi}sIgD^{low}$ phenotype while the rare B-1 cells of the spleen are $CD11b^-$ cells[121].

In mice, B-1 cells represent up to the 5% of the total B cells and represent a pool of long-living and self-renewing B cells that contribute to the production of the majority of circulating IgM[122]. Indeed, they are defined as "innate" B cells for their ability to produce most of the natural polyreactive Abs

against self- and foreign-Ags. These Abs have a low affinity and broad specificities and provide the first line of defence against infections. The restriction in the repertoire reflects the fact that specific Ag stimulation and somatic hypermutation are not required. For all these reasons, together with the functionally linked splenic MZ B cells, they represent a bridge between innate and adaptive immunity. These two B cells subset are indeed ontologically evolved to deliver responses for gut or peritoneal and blood-borne Ags, in a T-independent manner favouring the development of rapid responses to a restricted number of conserved Ags[123].

Even if they share many phenotypical characteristics, B-1a and B-1b are also functionally separate. For example, concerning humoral functions, B-1a cells are reported to constitutively release natural IgM Abs against *S. pneumoniae* while B-1b are able to make Abs specific to *S. pneumoniae* (producing a so-called "memory IgM")[124]. Moreover B-1a cell development is strongly influenced by B cell Ag receptor (BCR) specificity and strength of signalling as well as by the presence of an intact CD19. To date, robust BCR signalling in B-1 cells has been shown to be dependent on the positive regulation through the CD148-Lyn axis, while in B-2 cells CD148 covers a redundant role[125].

The definition of the developmental phases of the B-1 cells is intriguing and it is still a matter of debate. With elegant experiments, in 2006, the existence of a B-1 cell-specific progenitor in the Lin^{-} $CD45^{low\text{-}neg}CD19^{+}$ population was identified. This progenitor was indeed able to reconstitute only B-1 cells in SCID mice recipients. Previously the maturation of B-1 cells was reported to derive from fetal or neonatal precursors existing in mouse liver and omentum[126]. These authors, instead, also showed the presence of the B-1 progenitor in the adult bone marrow, suggesting the possibility that B-1 cell numbers during adult life can be supplemented by progenitor cell differentiation besides self-renewal potential[127]. The two existing models concerning B-1 cell development were reviewed by the authors in the same year, and shown in the *figure 7*.

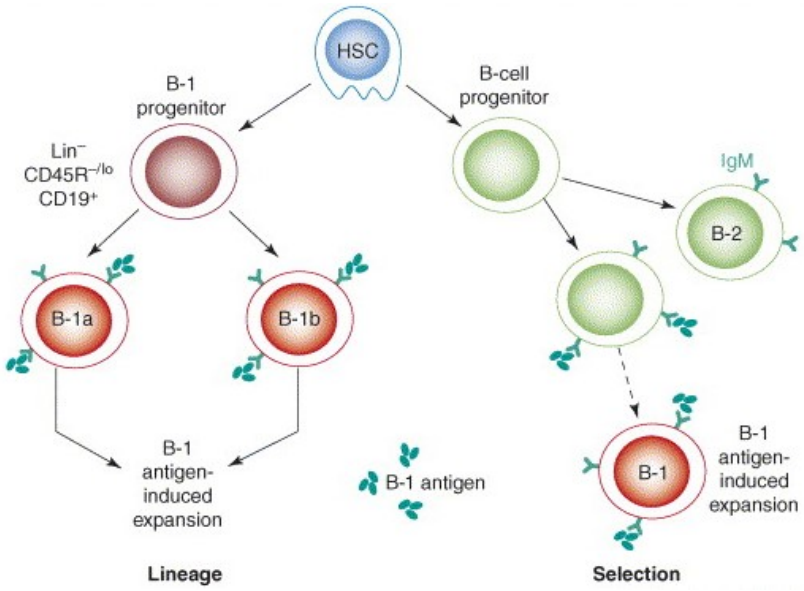


Figure 7: Models of B-1 cell development from an HSC. B-1 cells, alike all other blood cell type, derive from the HSC. The lineage model of B-1 cell development proposes the existence of a $Lin^{-}CD45^{-/low}CD19^{+}$ progenitor with the developmental potential to the production of B-1a and B-1b cells. The selection model instead proposes that B-1 and B-2 cells develop through a single developmental pathway, by a common B-cell progenitor, and that Ag-binding to cells at the sIgM-expressing stage of development determines whether a cell will acquire the characteristics of B-1 or B-2 cells[121].

Their finding of the $Lin^{-}CD45^{low\text{-}neg}CD19^{+}$ population in the fetal liver and fetal and adult bone marrow from which B-1 cells develop support the lineage model.

More recently, in 2011, the same authors investigated the nature of B-1 transition elements. They discovered the existence of a common B-1 lymphoid progenitor and that B-1 cells can derive from $sIgM^{+}CD93^{+}CD23^{+/-}$ transitional cell intermediates that predominate in neonatal spleen during the first two weeks after birth, proposing a new model for peripheral B-1 (and B-2) cells maturation[128,128] (*Figure 8*). In addition, they demonstrated that, both the inactivation of the classical pathway and the alternative NF-kB pathway block the appearance of B-1 and B-2 transitional cells. While the B-2 transitional cell maturation resulted to be dependent on the alternative NF-kB signalling, mature B-1 cells are reduced in mice carrying defects in the classical NF-kB signalling without affecting the maturation of transitional B-1 cells. On the contrary, the classical pathway is necessary for the maintenance of mature B-1 cells and in particular for B-1a cells.

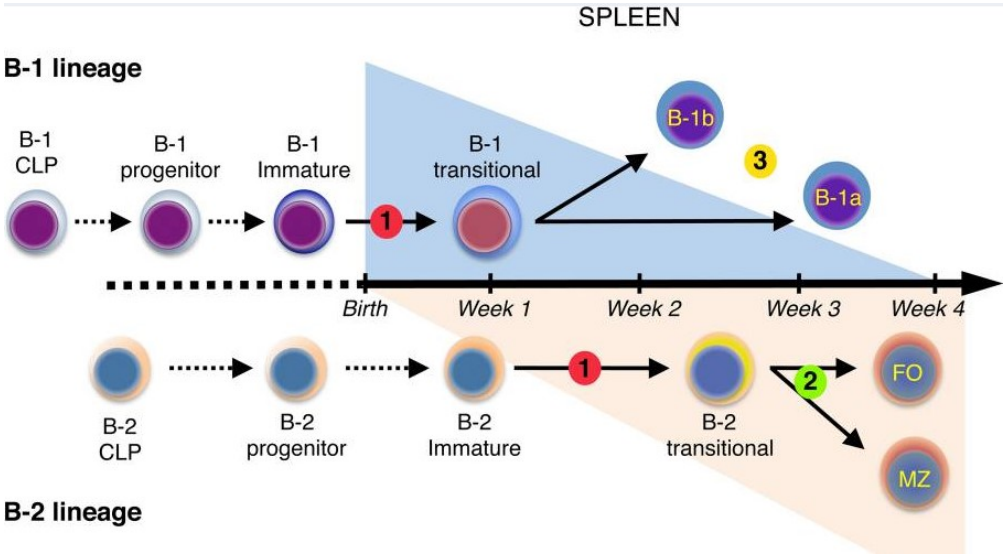


Figure 8: Model of layered B-1 and B-2 development. In a first neonatal wave, B-1 CLPs generate B-1 progenitors that differentiate into immature B-1 cells. These immature cells migrate to the spleen and there, after a B-1 transitional cell intermediates they mature into B-1a and B-1b cells. In a second wave, B-2 CLPs generate B-2 progenitors and then immature B-2 cells that form splenic B-2 transitional cells. This B-2 transitional cell wave is the predominant one in adults[129].

These results were afterwards confirmed and extended with the identification of phenotypical characteristics that are useful to discriminate transitional cells with B-1a potential from the B-2 counterpart. The transitional B-1a element is unique for the co-expression of CD93, IgM, CD43 and low levels of CD45R. Transitional B-1a cells are, in addition, $CD5^+$ and the majority of mature peritoneal cavity B-1 cells generated from these precursors are B-1a cells. Differently, neonatal Wt $CD93^+IgM^+CD5^-$ transitional cells predominantly mature into B-2 cells. This population also gives rise to some B-1b cells and even B-1a cells, showing an unappreciated heterogeneity of the neonatal transitional-cell population. The $CD5^+$ transitional B-1a cells derives directly from the $CD5^-$ progenitor. Moreover, the presence of an intact NFkB signalling via IkBNS in the transitional B-1a subset ,was essential for the development of B-1 cells[130]. These experiments were conducted in the *bumble mice*, a mutagenic mouse in which there is a complete disruption of the gene encoding the IκBNS protein belonging to the nuclear IκB-like family of proteins[131]. Indeed, previously, an impaired B cell development and function were shown in the absence of IkBNS[132]. This *bumble* mouse assumes, therefore, the phenotype of a B-1 deficient mouse model (having in addition also a severe reduction of the numbers of splenic MZ B cells). The heterozygous mice for the IkBNS mutation have instead a mild phenotype with reduced levels of circulating IgM Abs and responses to TI-2 Ags but normal frequencies of B-1 and MZ-B cells[133].

A *de novo* influx of B-1 cells in adult mice seems to be severely restricted under steady state conditions. The maintenance of the B-1 lymphocytes is favoured by their self-renewal capability, this process relies on cyclin D2. The constitutive release of high amounts of cytokines such as IL-10, IL-6, IL-5 and IL-9. IL-10 is mainly carried out by B-1a cells, that have also been identified as a regulatory B cell population; IL-5 instead is mainly produced by the B-1b subset[134].

Very recently the group of Karlsson Hedestam G.B. further investigated the possibility that neonatal spleen is required for B-1a cell development. They observed that neonatal splenectomized mice had similar peritoneal cavity B-1a cell frequencies to those observed in control mice until 6 weeks after surgery. They propose that an intact spleen is essential for B-1a cell maintenance rather than generation. They used a model in which fetal liver cells were transferred from pre-splenic embryos into splenectomized recipient mice. Moderately lower numbers of B-1a cells developed in asplenic mice. B-1a cells were then generated in the absence of the spleen but the neonatal spleen was instead essential for the maintenance of the B-1a compartment[135].

In B-1 cells, whose BCRs are cross-reactive to self-Ags, inhibitory mechanisms were developed in order to prevent their activation in the absence of infection and autoimmunity. Differently from B-2 cells, BCR ligation induces modest calcium mobilization, little or no proliferation, and increased apoptosis. CD5, that is in the complex with IgM, negatively regulates the BCR signalling in peritoneal cavity B-1 cells. Experiments conducted in Wt and CD5 K.O. mice showed that the proliferation of both mice derived B-1 cells was similar in response to anti-CD40 and LPS stimulations. Instead, proliferation to anti-IgM was effective only in CD5 K.O. B-1 cells and the blocking of CD5 association with mIgM recovered the proliferative defect upon BCR ligation[136]. Later, Lyn, and SFK were reported to negatively regulate the BCR signalling by phosphorylating the ITIM motifs in B cell co-receptors[137]. The high calcium mobilization after co-cross-linking of BCR and CD19 that occurs in B-2 cells, is defective in both B-1a and B-1b cells that are equally hypo-responsive to synergistic stimulation[138]. The mobilization of mainly extracellular calcium reflects these defects in calcium response. Finally, autocrine IL-10 production mediates also autoregulation in controlling the expansion of self-reactive B-1 cells[139].

3.2.1.2. B-2 B cells

B-2 cells in the mouse are the predominant population of B cells in the spleen, LNs, and peripheral blood. After birth B-2 cells are continuously replenished by bone marrow hematopoiesis and stroma

cells, provides all the necessary signals for the commitment of the progenitors. Functionally different from B-1 cells, B-2 conventional lymphocytes participate strongly in adaptive immune responses providing support to Ag-specific T cell activation and generating memory cells and plasma cells (PCs) able to recognize ad attack more efficiently previously encountered pathogens.

In the differentiation process, pluripotent HSCs originate naïve FO or MZ B cells through a complex and highly regulated process. Soluble signals and cell-to-cell contacts such as IL-7 and Fms-like tyrosine kinase 3 ligand (Flt3L) derive from bone marrow stroma cells. The differentiation process involves intermediate developmental stages identified by the expression of specific transcription factors and surface molecules, Ig heavy and light chain rearrangement and expression of B cell receptor complexes[140]. Multipotent progenitors lose their self-renewal capacity and express Flt3, that is fundamental for the growth and survival of the progenitors[141]. According to the classical view, the MPP generate two lineage-restricted populations, named CMPs and CLPs. In 2010 an alternative myeloid-based model of hematopoiesis has been proposed: it was suggested that myeloid potential persists in the T and B cell branches, even after the divergence of the two lineages[142]. PU.1 and Ikaros are the two transcription factors expressed in MMPs that control Flt3, c-Kit and IL-7R alpha signalling; PU.1 is determinant in the commitment of the myeloid or lymphoid fate[143]. CLP commitment to the B cell lineage depends again on specific transcription factors: the absence of E2A, early B cell factor 1 (EBF1), and paired box protein 5 (Pax5) blocks B cell maturation (also shown in *picture 9*).

The pre-pro B phase is characterized by the expression of CD45R, the lack of CD19 a no Ig rearrangement. In the pro-B stage recombination-activating gene-1 (RAG-1) and RAG-2 recombinases are up-regulated and the process called V(D)J recombination, that allows rearrangement and recombination of the Ig genes, can take place[144]. During the gene rearrangement of the receptor the variable (V), diversity (D) and joining (J) gene segments an huge repertoire of different Ag receptors is generated. In the early phase of pro-B stage D_H-to-J_H genes are rearranged, in the late phase of pro-B, the joining of a V_H segment to the pre-arranged D_HJ_H complex occurs. A successful rearrangement of the heavy chain gene on one chromosome represses the rearrangement of genetic material from the second chromosome. Afterwards, a mechanism called allelic exclusion guarantees the monoallelic expression of the IgH gene and monospecific Ag recognition[145]. After heavy-chain rearrangement the cell expresses the pre-BCR in which the rearranged μ heavy chain is combined with the surrogate light chains λ5 and VpreB; moreover, the pre-BCR associates with the Igα/Igβ dimer (CD79a/CD79b dimer), this cell is now called the pre-B

cell. The expression of a functional pre-BCR is a critical step, it initiates cell cycling and light chain rearrangement in the small pre-B cells that undergo V-J joining on one light chain chromosome; the resulting light chain must be able to pair with the μ heavy chain in order to form a functional BCR. It is important to note that alterations of its functionality have been associated with cancer and immunodeficiencies. Cells expressing functional BRC have acquired Ag specificity and have reached the stage of immature B cells.

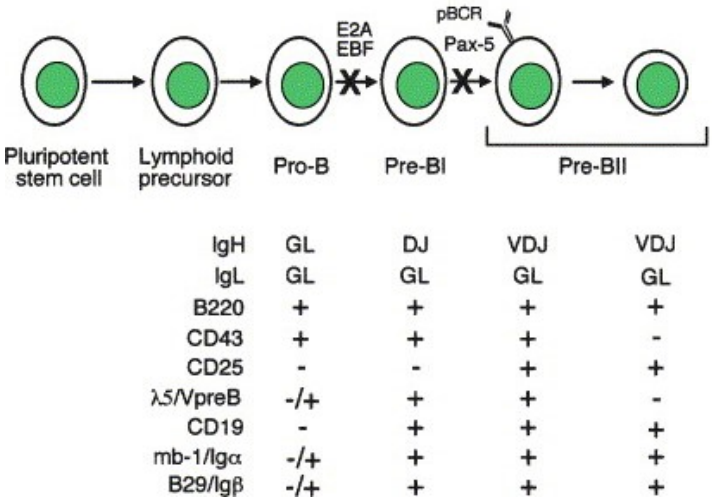


Figure 9: B cell developmental stages from the pro-B to the immature B cell stage. Each stage of B cell maturation is defined by the rearrangement status of the Ig_H and Ig_L chains. The X indicates the stages in which the absence of the E2A, EBF and Pax-5 induce arrest of the development. Each stage is also defined by the expression of surface markers and status of V(D)J recombination. Adapted from[146].

If from one side this reflects the ability of B cells to respond with several different Ab specificities, on the other these receptors can react against self-Ags. To prevent this risk, the immune system developed two important mechanisms: the negative selection of lymphocytes that express high affinity receptors for *self*-Ags triggering these cells to apoptosis, and the receptor editing, i.e. the capacity to reactivate the Ab recombination process in order to express a receptor no longer specific for a *self*-Ag. There is also an intrinsically regulated mechanism that brings these auto-reactive clones to a state of anergy. Moreover, extrinsic controls can inhibit cells that evaded upon itemized mechanisms limiting the supply of essential growth factors, co-stimuli, pro-inflammatory mediators[147]. Immature B cells that survive negative selections egress from the bone marrow and specifically home the spleen where a transitional developmental stage takes place in order to become mature naïve B cells. B cells are naïve until the encounter with an Ag, that induce the maturation into FO or MZ B cell.

Two main types of transitional cells can be identified: transitional 1 (T1) or newly formed (NF) B cells that express IgM, very low levels of the complement receptors CD21 (that is part of the CD19-CD21-CD81 co-receptor complex) and do not express follicular markers such as IgD and the low affinity receptors for IgE, the CD23. T1 cells mature into transitional 2 (T2) B cells that form follicles and are able to recirculate; moreover they acquire the expression of surface IgD and CD23 and express intermediate levels of CD21[148]. The next fate of a transitional cell is to mature into either FO or MZ B cell: the strength of the BCR signalling and the participation of Notch2 lead to one or the other B cell splenic subset. If the BCR reacts with an intermediate affinity with a self-Ag it is induced to differentiate into a FO B cell while if it reacts weakly to the *self*-Ag signals drives it to an MZ B cell fate[149]. These phases of splenic FO maturation are regulated by the presence of specific factors such as B cell activating factor (BAFF). This is a member of the TNF superfamily that through BAFF-receptor (BAFF-R) mediates survival of B cells involving mainly non-canonical NF-κB activation. However, canonical NF-κB signalling is also required for MZ B cell development and survival[149]. The transitional developmental phase is shown in the *figure 10*.

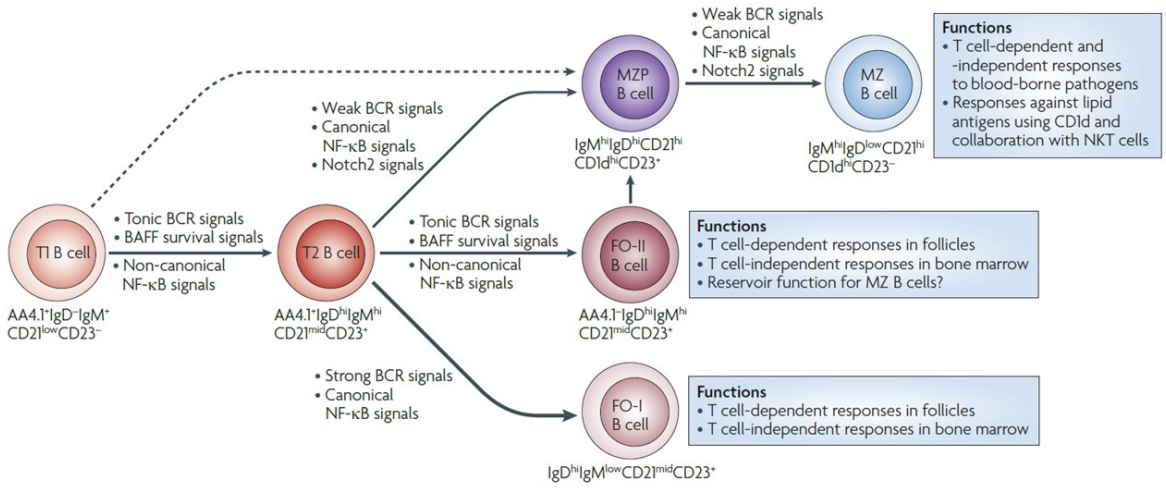


Figure 10: Model for transitional B cells maturation. In the spleen transitional (T1 and T2) B cells can mature in either follicular (FO) or marginal zone (MZ) B cells. This cell fate decision depends both on the strength of BCR signalling and the involvement of Notch2 signals. It is probable that T2 B cells mature into FO B cells if they recognize *self*-Ag with a high affinity, whereas *self*-reactive B cells with a lower affinity can mature into MZ B cells. BAFF survival signals are also important in this process[149].

FO B cells are the main splenic B cell population and they are appropriately located to perform T cell-dependent immune responses in the secondary lymphoid organs. MZ B cells represent a sessile 5% of B cells and after maturation migrate to the white pulp of the spleen, a definite anatomic location between the marginal sinus and the red pulp. The MZ B resemble for many aspect B-1 cells:

they have potential to self-renew and can survive indefinitely, express polyreactive BCRs and high densities of TLRs and are able to initiate rapid T cell independent and T cell dependent Ab responses against blood-borne bacterial Ags. Phenotypically FO B cells are $IgM^{hi}IgD^{low}CD21^{+}CD23^{+}$. The levels of CD21 expression helps to distinguish into FO type I that are $CD21^{mid}$, while FO type II are $CD21^{hi}$. MZ B cells are instead $IgM^{hi}IgD^{low}CD21^{hi}CD1d^{hi}$ and $CD23^{-}$[150]. Therefore, the use of the markers CD21 and CD23 helps to discriminate the two B populations. Moreover MZ B cells, similarly to B-1 cells, largely express CD9 and also this surface molecule is suggested as a new marker to distinguish MZ from FO B cells[151]. It is also worth noting the expression of CD1d, a non-classical MHC class I molecule that allows them to present lipid Ags to the invariant natural killer T cells (iNKT). CD1d expression identifies uniquely MZ B cells, a marker that is also suggested to be a marker of regulatory B cells[152].

3.2.2. B cell activation

For their activation B cells need to meet an Ag and to recognize it through the BCR at the level of peripheral lymphoid organs. In this process, the Ag induces Ig receptor aggregation leading to a signal cascade and, "second" signals, notably cytokines, chemokines or growth factors lead to B cell activation. Activated B cells differentiate either into PCs, Ab-secreting cells, or memory cells that are responsible for long-term protection against secondary infections[153]. Depending on the nature of the Ag and the type of the second signal two types of B cell activation can be distinguished: T cell-dependent (TD) and T cell-independent (TI).

3.2.2.1. T cell-dependent activation

TD activation involves Ags with non-repeated motifs that are not able to trigger an Ab response alone, for instance, B cells need the activity of helper cells. Indeed, the second signal required to induce the Ab response to these Ags is delivered by Th cells that recognize Ag fragments bound to MHC-II molecules presented by the B cells. This recognition induces from one side cytokine secretion by Th cells and a stronger physical co-stimulation through the axis CD28-B71/2, CD154(CD40L)-CD40 or CD134(OX40)-OX40L respectively in the T-B cell interaction; in turns, this produces B cell proliferation and a process called class switch recombination (CSR)[154]. The CD40-CD40L co-stimulatory system plays a central role in B cell activation, inducing processes such as proliferation,

clonal expansion, Ab production, GC formation, isotype switching, affinity maturation and induction of memory cells[155].

Ag-activated B cells can differentiate into short-lived PCs or enter B cell follicles in secondary lymphoid organs forming GCs. The GCs maturation involves mainly FO B cells but also MZ and B-1 B cells can respond to a TD activation with the formation of extrafollicular PCs[156]. GCs are structures in which somatic hypermutation (SHM) takes place: this important process allows the establishment of mutations on the variable regions of Ig genes producing high variability in the Ag recognition. Mutated GC B cells migrate in the light zone of the follicles. Here, favourable mutations are positively selected through the interaction with Th cells and follicular DCs. Negatively selected B cells undergo apoptosis.

Positively selected GC B cells can undergo CSR. Mature B lymphocytes express on their membrane IgM Igs, with the μ heavy chain, and IgD, endowed of δ heavy chains, both with the same variable Ag binding regions. This process consists in changes in the Ig heavy chain constant region (C_H) gene from C_μ to one of the other C_H genes and this results in a switch of the Ig isotype from IgM or IgD to either IgG, IgE or IgA. The isotype is determined by the manner in which Ags are eliminated and the localization of delivering and accumulation the Igs[157].

A very important enzyme that is expressed in GCs by activated B cells is the so-called activation-induced cytidine deaminase (AID) enzyme. It belongs to a group of mutagenic proteins of apolipoprotein B mRNA-editing catalytical component (APOBEC) family and is essential for initiating both SHM and CSR. It has been shown that AID deficiency causes the complete absence of class switching in mice before or after immunization with TD Ags[157].

Memory B cells and PCs, that are positively selected in the GCs, are long living cells that localize in specific microenvironments. Memory cells preferentially homes in secondary lymphoid organs, while PCs the bone marrow that provide survival and proliferating factors most of them belonging to the TNF family of ligands. Among them it is important to remember BAFF, that maintains B cell homeostasis by acting as a survival factor for transitional B cells, and the strictly related proliferation-inducing ligand (APRIL) cytokine. APRIL acts at a later stage, modulating the functions of Ag experienced B cells. These critical factors in the maintenance of the B cell pool and humoral immunity are also involved in several autoimmune diseases with autoreactive B cells such as systemic lupus erythematosus (SLE) and **tumors** (e.g. multiple myeloma) and are target of many therapeutic drugs[158].

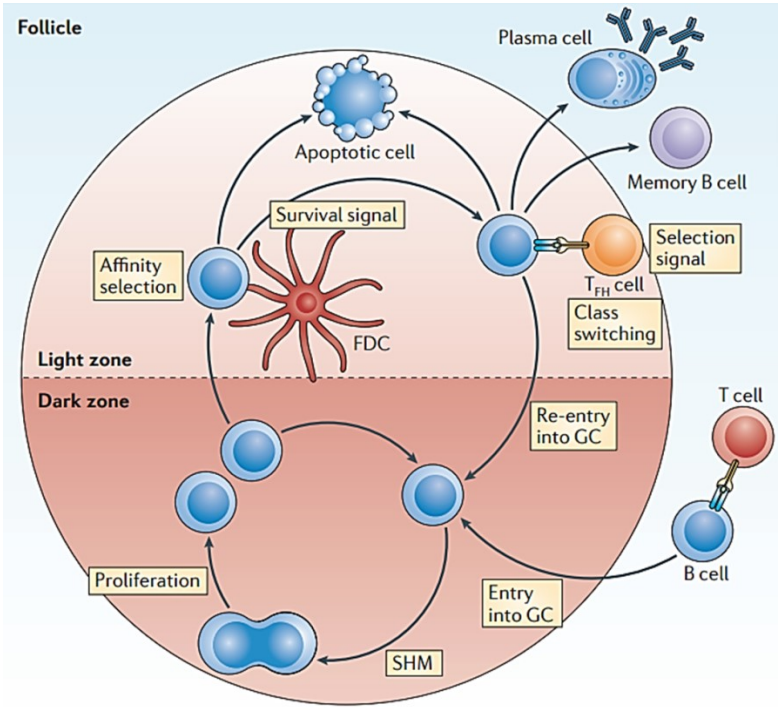


Figure 11: GC reactions. In a TD GC reaction B cells present Ag to T helper cells and receive co-stimulatory signals. These B cells enter the dark zone of the follicle and undergo SHM by upregulating the AID enzyme. After proliferation B cells migrate to the light zone. Here the mutated BCRs are exposed to Ags that are exposed to immune complexes on the follicular dendritic cells (FDCs). If the affinity of the BCR is extremely low, the B cell will face apoptosis. T helper cells favour the survival of higher affinity B cells and induce the others to undergo apoptosis. Surviving B cells can then have three possible fates: they can re-enter the dark zone and go through further proliferation and SHM, they can exit the GC as plasma cells or they can exit as memory B cells[159].

Excursus: Ab isotypes

The generation of Ig diversity happens during B cell development and GC reactions and is a hierarchical process. In mammals, five main classes of Abs can be identified: IgM, IgD, IgG, IgA and IgE. The differences among the isotypes concern the sequence and number of constant domains, hinge structure and the valency of the Ab. The role of the Abs is to neutralize their targets preventing their entrance in the host cells, to activate immune cells by binding to Fc receptors (FcRs) and activating the classical pathway of the complement system by binding to C1q. The effector mechanism is regulated by the heavy-chain isotype and binding affinities of activating and inhibitory FcR on immune cells. For example, IgM and IgG3 are excellent complement activators, while IgG1 and IgE bind FcR to activate macrophages and MCs respectively[160].

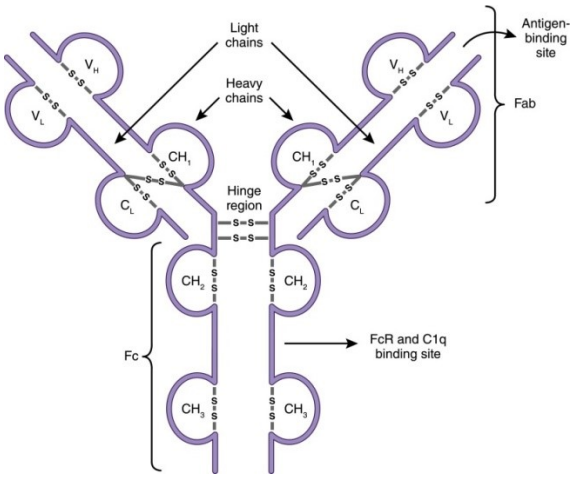


Figure 12: Ab structure. Igs are made of two heavy chains (VH and CH) and two light chains (VL and CL). The Ag-binding fragment, Fab, is composed of one variable domain from each heavy and light chain (VH and VL). The variable domains contain the complementarity determining regions (CDRs) with the most sequence variations and determine Ab specificity. The constant domains CH2 and CH3 of the heavy chain make up the crystallizable fragment, Fc, which mediates effector functions through binding to Fc receptors (FcRs) on cells and to complement (C1q) [160].

IgM is the first type of Ab to be produced during an humoral immune response since it can be expressed without isotype switching and before SHR. IgM are therefore low affinity Abs, but they form pentamers whose ten Ag-binding sites can simultaneously bind to multivalent Ags such as bacterial capsular LPS, compensating the relatively low affinity with high overall avidity. Because of their size IgM are mainly found in the blood; they are very effective in activating the complement system and innate like-B cells such as B1. MZ B cells are mainly producers of this isotype.

IgG isotype is mainly found in the blood and extracellular fluid, while IgA is the principal isotype in secretions, especially in the mucus epithelium of the intestinal and respiratory tracts. IgG efficiently opsonizes pathogens for engulfment by phagocytes and activates the complement system, IgA is a less potent opsonin and a weak activator of complement. The importance of IgA in mucosal immunity will be further discussed in *section 3.3.2 "Mucosal immunology of the intestine*".

IgE Abs are present at very low levels in blood or extracellular fluid, but are bound avidly by FcεRI on MCs that reside in the skin, in the mucosa and along blood vessels in connective tissues[161].

3.2.2.2. T cell-independent activation

TI activation is mainly triggered by microbial products that do not have a protein nature and can directly activate B cells without the need of Th cells, allowing rapid responses to many bacterial and viral pathogens. In this case, the second signal can be provided by a direct recognition of a common microbial constituent or by an accessory cell belonging to the innate system. These Ags possess repetitive and conserved structures and are divided into two categories.

TI-1 Ag, also named mitogens of B lymphocytes, at low concentrations drive a specific Ab response while at high levels they cause a polyclonal B cells activation. One classical TI-1 Ag is the LPS that drive B cell activation via TLR4. LPS induces B cell proliferation and differentiation into Ab secreting cells and stimulates IL-6 secretion in mature B cells through PI3-K signaling pathways[162]. In men B cells lack TLR4, but alike in mice, express TLR9 that promotes proliferation, IgM and IL-6 production in response to the CpG DNA[163].

TI-2 Ags are characterized by repetitive structures and present high molecular weights. They are able to activate mature B cells thanks to the their capability to aggregate the BCR on the cell membrane. In particular, these Ags affect mostly B-1 and MZ B cell populations and induce their production of polyreactive and low affinity IgM Abs in the absence of a strong second signal or the establishment of GC reactions[164].

3.2.3. B cells: more than Ab-producing cells

B lymphocytes are the only immune population capable of differentiating into Ab-secreting cells, however their importance goes far beyond this humoral function, for instance they can efficiently present Ags to T cells and produce a large plethora of cytokines.

There are evidences supporting the contribution of B cells in the organization and remodelling of secondary lymphoid tissues in naive mice and after immune activation. It has been shown that B cells provide important factors that favour lymphoid organogenesis, such as the lymphotoxin α1β2 (LTα1β2) and the TNF-α in the spleen, LNs and Peyer's patches. A bidirectional interaction is established between B cells and stroma cells favouring the development of FDCs (that in turns produce CXCL13 that recall B cell in the follicles and induce their production of LTα1β2) and macrophages.

Different cytokines produced by B cells such as TNF, IFN-γ and CCL3 have been show to affect in many stages the polarization of $CD4^+$ T cells in lymphoid compartments. The differentiation of Th1 and Th17 cells was shown to be promoted by B cell-derived IL-6 in experimental autoimmune encephalomyelitis (EAE). In mouse models of atherosclerosis, pulmonary infection and sepsis, some $CD138^{hi}$ PCs were observed producing GM-CSF that was responsible for increased IL-12 production by DCs and therefore being essential for polyclonal IgM secretion by B-1 cells after microbial stimulation. All these examples are reviewed in[165].

Beyond active roles in the clearance of infections and T cell polarization, B cells are also known to cover important roles in the dampening of immune responses. First evidences that B cells could suppress inflammation by the provision of IL-10 emerged in models of colitis, EAE, and arthritis were published[166,167,168]. Unlike the expression of a common identified marker for Treg, the Foxp3 transcription factor, there is no agreement on a specific phenotype for Breg, indeed multiple subsets of IL-10-producing Breg cells have been described. At the present moment, the most reliable hypothesis is that in response to certain stimuli, B cells take on a regulatory phenotype to suppress local inflammation[169]. The CD40 activation, stimulation through TLRs, the activation with IL-6, IL-21, IL-1β, IL-35 and BAFF cytokines are reported to induce a regulatory phenotype in immature B cell[170]. Many works conducted both in mice and humans have attributed their suppressive role to the capacity to produce IL-10 but there are also evidences that TGF-β and IL-35–producing murine B cells also exert regulatory functions[169]. Breg cells play a protective role in autoimmune diseases such as allergy, RA, SLE, multiple scerosis, and EAE, in which instead the proinflammatory Th1 or Th17 profiles show detrimental effects in affected individuals. On the other side, therapeutic inhibition of Breg cells can have helpful effects in the progression of some cancers and infections[171].

3.2.4. Features of B cell chemotaxis in health and pathology

Chemokines direct all the phases of B cell biology, starting from the maturation in the bone marrow (or from the liver in the fetal life), driving their physiological egress and the localization in secondary lymphoid organs where they undergo activation. They play a fundamental role in the homeostatic trafficking of B lymphocytes and are also important modulators of B cell chemotaxis during infections and pathological conditions such as tumors. For this reason, physiological and pathological B cell-related chemokines can be distinguished. A list of mouse B cell-related chemokines that will be considered in the results of this thesis is reported in the *table 2.*

Table 2: Examples of B cell-related chemokines and receptor in homeostasis and inflammatory conditions
*the CCL20/CCR6 axis covers importance in both physiological and pathological processes
It is important to highlight that these receptor-ligand couples are not B-cell-specific since they are differently shared with other lymphocytic population such as T cells and innate immune cells (monocytes, DCs, macrophages, MCs, iNK cells…).

Homeostatic B cell chemokines		**Pro-inflammatory B cell chemokines**	
ligand	**receptor**	**ligand**	**receptor**
CXCL12	CXCR4	CCL2	CCR2
CXCL13	CXCR5	CCL5	CCR1,3,5
CCL19, CXCL21	CCR7	*CCL20**	*CCR6**
CCL25	CCR9		
CCL28	CCR10		
*CCL20**	*CCR6**		
CCL17, CCL22	CCR4		

The chemotactic properties of B-2 and B-1 cells from their development are described here below. Changes occurring during pathological processes are also mentioned with a particular attention to the CCR6/CCL20 pair that is an important bridge between homeostatic and inflammatory conditions and is of great relevance for the results obtained in this work.

Retracing the early B cell development, CXCR4 expression on B cells, the receptor for CXCL12, covers an essential role. Indeed, the lack of this receptor or of its ligand drastically blocks B lymphopoiesis. In the bone marrow the retention of B cell precursors is favoured by the CXCR4-CXCL12 axis[172]. Moreover, CXCL12 has been shown to be required also for the development of the B cell precursors in fetal liver[173].

At the stage of pre–pro B the cells are responsive mostly to CCL25, the ligand to CCR9. Subsequently, at the level of pre- and immature B cells, these lymphocytes become first more receptive to CCL19, CCL21 (ligands of CCR7) and after they acquire responsiveness CXCL13 through the increased expression of CXCR5. This last receptor favours the migration of immature B cells into CXCL13-expressing B cell follicles[174]. One interesting observation is that even though in the MZ of the spleen (adjacent to the B cell follicles) CXCL13 is not expressed, MZ B cells possess a similar migration capacity to CXCL13 compared to FO B cells *in vitro.* Indeed, after a pro-inflammatory stimulation, MZ B cells are able to promptly migrate to B cell follicle in a CXCR5-CXCL13 dependent manner. In this regard, it has been shown that under physiological conditions, MZ B cells receive a retention

signal triggered by sphingosine 1-phosphate (S1P) suggesting an inhibition of motility and accumulation of MZ B in B cell follicles while during inflammation there is a reduction of the S1P signalling in MZ B cells[175].

B-1 cells, that are closely related to MZ B cells, also have the ability to migrate to mucosal sites, especially in the gut mucosa, where they are reported to secrete polyvalent IgA Abs, always in a TI-fashion. To date, up to the 40% of plasma and intestinal IgA is considered to have a B-1 origin and to be IL-5 dependent[176,177]. The CXCR5-CXCL13 axis is fundamental for B-1 cell homing, regarding their retention in the peritoneum, high levels of integrins and tetraspanin CD9 are essential. When B-1 cells egress from the peritoneal cavity CD9 down-regulation occurs, a process that is favoured by stimulation of TLRs among which the LPS is one of the most relevant[178]. Peritoneal cavity B-1 B cell migration in the intestine is also influenced by S1P since its deficiency alters the composition of peritoneal cavity B cell populations and reduces secretory IgA levels. S1P is suggested as a target to affect the migration and activation of peritoneal cavity B-1 B cells in pathological conditions such as IBD and sepsis where they are reported to play a role[179].

Upon activation, a conventional B cell can become a long-living memory or PCs. IgG PCs express high levels of CXCR3 and the integrin $\alpha_4\beta_1$, allowing their retention in non-mucosal sites; moreover they express high levels of CXCR4 that is responsible for their homing in the bone marrow[180].

PPs are the main site of B-cell activation in response to intestinal Ags, leading to the generation of gut-homing plasmablasts that produce secretory IgA and attract great numbers of circulating B cells. IgA-secreting PCs are activated in mucosal tissues and aero-digestive tract. Their mucosal homing is favoured by the expression of $\alpha_4\beta_7$, CCR9 and CCR10. There are however some specificities: CCR9 allows the selective localization in the colon, mammary gland and respiratory tract; the small intestine, instead, is colonized by both $CCR9^+$ and $CCR10^+$ IgA^+ PCs, so CCR9 and CCR10 have overlapping roles in localization of these cells to the small intestine. Interestingly, higher levels of circulating $\alpha_4\beta_7{}^+CCR10^+$ plasmablasts have been detected in patients with acute colitis compared with healthy subjects[181].

Naïve and memory B cells express CCR6 and are selectively responsive to its ligand CCL20. The expression of this receptor is not fundamental to induce their proliferation but is essential in their ability to respond to a recall response to their cognate Ag[182]. Its expression on B cells is increased after a BCR-, CD40+IL-4- or LPS-dependent activations but is drastically decreased in PCs. B cells from CCR6 K.O. mice produce Abs with lower affinity, suggesting that CCR6 influences GC dynamics and is important in the entry of B cells into the GC reactions[183]. In the PPs, where the expression of

CCR6 and CCL20 is high, CCR6 deficiencies have been shown to impair TD-IgA production that lead to a dysregulate antimicrobial peptide production and an imbalanced intestinal microbiota[184].

CCR6 is involved also in pathological processes, for example, men suffering from IBD and in murine models of colitis present higher levels of CCL20 compared to uninflamed colon tissues. Ab treatments against CCL20, or the desensitization to CCR6, are described to inhibit adhesion of T and B cells to inflamed microvessels in mice with dextran sodium sulphate (DSS)-induced colitis[185]. This axis has also been lately investigated in autoimmune conditions, where autoreactive B cells play a dominant pathogenic role. In SLE CCR6 has been shown to be up-regulated in patients; moreover in the pathogenesis of RA, the accumulation of autoreactive B cells within inflamed synovia, was seen to be synergistically favoured by CXCL13 and CCL20[186]. CCL20 augmented expression is also studied in several cancer types such as hepatocellular carcinoma, colon cancer, breast and ovarian cancer, thyroid cancer, pancreatic cancer, melanoma and many others[187,188]. As an example, in the hepatocellular carcinoma, tumor derived-CCL20 interacts with CCR6 highly positive $CD5^+$ B cells. In this work, conducted both in humans and mice, the recruitment of the B cells favoured cancer progression by enhancing angiogenesis[189].

The most widespread barrier to the external environment is the skin, which is indeed rich in both innate ad adaptive immune cells. During dermis infections, autoimmunity or cancer, an accumulation of B cells in the skin is observed while in homeostatic conditions B cells are almost absent. However, in the last years B cell trafficking in the skin during homeostasis has been reconsidered and the most known skin-homing chemokine receptors for lymphocytes are CCR4 and CCR10 and CCL17 and CCL28 their respective ligands. As an example, Geherin et colleagues showed the presence of B cells in both uninflamed skin-draining afferent lymph and the uninflamed flank skin; in addition, in a model of granulomatous skin inflammation they observed that the greatest relative increase of lymphocyte subsets was the one B cells. In their work, they identified B-1-like cells recirculating through the skin and they showed that skin draining B cells are spontaneously responsive to CCL20, thus proposing the CCR6-CCL20 axis as a candidate for inducing B cell localization to skin[190]. The existence of innate-like IL-10-producing B cells in the skin of both mice and humans was later confirmed. A peritoneum–skin migratory axis for $\alpha_4\beta_1^+$ B-1 cells has also been suggested, in this work B-1 cells in a context of chronic skin inflammation, were playing a regulatory role[191].

3.3. MAST CELLS AND B CELLS IN HEALTH AND DISEASE

As we already said above, one of the most interesting features of MC biology is its "sociality". MCs are indeed predisposed to the cross-talk with other cell types thanks to the expression of several membrane co-stimulatory molecules and receptors and their capability to release a plethora of soluble mediators that can shape the behaviour of almost all other immune and non-immune cell types. The advantage of colonizing nearly all the vascularized body tissues allow them to interact with phenotypical and functional different cells such as B lymphocytes.

3.3.1. MC-B cell cross-talk: what is known and what is missing

In 1993, the first evidence of an interaction between these two cell types was reported. Specifically, Gauchat showed the expression of CD40L on both MCs and basophils and demonstrated that in the MC-B crosstalk, the axis CD40-CD40L, in conjunction with the presence of IL-4, was sufficient to promote IgE synthesis in B cells. The importance of this discovery lies on the fact that isotype switching could take place not only in LNs GCs but also in peripheral organs[192]. In 1996, BMMCs were shown to be able to induce resting B cells activation: the effect was shown after culture of the two cells and resulted in B cell proliferation and IgM production[193]. Another study appeared in 2002 when Skokos and collaborators showed that MC-derived exosomes are enriched in co-stimulatory molecules such as MHC-II, CD86, CD40L, LFA-1 and ICAM-1 that are able to drive B cell activation[111]. Afterwards, the following reports on a relationship between MCs and B cells were observed in inflammatory processes involving secondary lymphoid organs (e.g. LNs, tonsils) where the numbers of MCs increased compared to their physiological levels, suggesting an active role of MCs in driving adaptive immune responses. Moreover, increased numbers of MCs have been reported also in B-cell neoplasms including Hodgkin lymphoma, diffuse large B-cell lymphoma, lymphoplasmacytic lymphoma, and chronic lymphocytic leukemia[194,195]. In 2010 in Pucillo's group, the mechanisms responsible for the MC-dependent B cell survival and proliferation were deeply investigated. They showed the contribution of both resting and activated BMMCs in B cell survival and proliferation. Cell-to-cell contact through the CD40-CD40L axis and the release of soluble mediators by activated MCs resulted to be synergistic. In addition, the CD40 expression on B cells was favoured in the setting of this interplay. Finally, both membrane contact and MCs' soluble mediators, such as IL-6, were involved in the expansion of $CD138^+$ and IgA-producing PCs. In the same work, evidences

concerning the close contact of IgA$^+$ PCs and infiltrating MCs were shown in human samples of intestinal mucosa of patients suffering from IBD[195].

Later, in the same group, a novel mechanism involving the CD40-CD40L axis was reported in the expansion of IL-10-competent B cells, evocative of a B cell-dependent regulatory potential. Interestingly, by using a Kit-deficient animal model they showed that, in tissue-specific microenvironments such as in the gut, the absence of MCs was paralleled by a reduced proportion of IL-10 competent B cells[196].

In the figure below the main roles of MCs in the regulation of B cells' biology are illustrated.

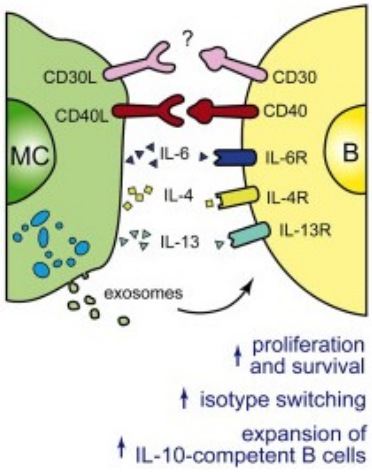


Figure 13: MC's derived soluble mediators and the physical contact through the CD40L-CD40 axis favours B cell activation and functionality. Signals derived from the CD40 signalling and the stimulation through IL-6, IL-4 and IL-13, or exosome-carrying co-stimulatory molecules, induce, in different combinations and diverse microenvironments, either survival and activating signals that lead to Ab switching or sustain the amplification of a regulatory IL-10-competent B cell population .

In literature, we can also find more recent reports concerning MC-B cell interaction analysed in different pathological process. In 2015, Kim and collaborators proposed a negative role played by IL-10-producing CD5$^+$ splenic murine B cells in dampening MC's allergic activation. They observed both *in vitro* and *in vivo* a reduced FcεRI signalling in the presence of splenic CD5$^+$ B cells-derived IL-10 [197]. In 2016 another group showed that MCs are able to induce the L-selectin up-regulation on B cells, suggesting a role in their homing properties, and of CD19, MHC-II and CD86 molecules, decreasing the threshold of activation of B cells and enhancing their Ag-presenting capacity. In their study, they also extended the role of MCs in B cells' isotype switching in the sustain of IgG production. Moreover, they investigated the impact of MCs on FO and MZ B: they observed that the effect on L-selectin modulation is higher on MZ, and that there is a larger effect on the Ab production while the impact on MHC-II and CD86 expression was more relevant on FO B cells[198].

The MC-B cell axis was also identified in a model of pulmonary hypertension. In a gene array analyses, the authors revealed that several components of Igs were among the most regulated MC-dependent genes. In agreement with this analysis, they detected increased circulating IgG after the infiltration of MCs into the hypertensive lungs. They propose that during the initial phases of the onset of pulmonary hypertension, MCs accumulate in the lung perivascular space and release their stored mediators pro-inflammatory factors, suggesting a role for IL-6. B cells in turn promote endothelial dysfunction resulting in vascular remodelling and pulmonary hypertension through an auto-Ab- mediated response[199]. A recent report was aimed at analysing IgE responses during helminth infections in RAG1$^{-/-}$ mice, appropriately reconstituted with specific populations of lymphocytes. They showed that, during helminth infections, B-1 cells make large amounts of IgE in a T-cell-dependent manner. This IgE production was enhanced by IL-25, that is increased following parasite infection. Moreover, this mechanism inhibits the IgE production by B-2 cells and, interestingly, were unable to induce MCs' degranulation and parasitic clearance proposing a regulatory mechanism that inhibits MC's functions. They demonstrated that helminth clearance by B-2- IgE cells was instead MC-dependent[200].

All the cited works highlight the importance of MCs in promoting the activation of conventional B cells in different physiological and pathological contexts. However, many aspects of this interplay still remain to be determined. A little attention has been put in characterizing the cross-talk comparing different B cell subtypes, such as the B-2 and B-1 lineages. Moreover the effect of B cells on MCs subtypes in allergic, autoimmune or cancer conditions still are unexplored fields.

3.3.2. Mucosal immunology of the intestine

Many of the works regarding MC-B cell interplay that are done in *in vivo* contexts involve the mucosal sites of the body. MCs indeed, as sentinels of the microenvironment are strategically located in close proximity not only with the blood vessels and nerves, but also close to the barriers that separate the organism with the external environment. Among the barriers of our body, the intestinal mucosa is maybe the most developed and is an important reservoir of tissue resident MCs.

How homeostasis is maintained in the intestinal mucosa

The gastrointestinal tract of mammals can be defined as the *non-self* environment of the body *par excellence* because of the continuous presence of food Ags and a tremendous number of microorganisms. For this reason, evolution has developed a complex system of barriers that, together with immune tolerance, are aimed at preventing activation and continuous inflammation. The system of resident bacteria, fungi and viruses that evolved in symbiosis with mammals form the gut microbiota. The microbiota offers several benefits to the host: it synthesizes the B and K vitamins, produces metabolites such as the short-chain fatty acids (SCFA), which is a source of energy and enhances the mucus production. SCFA, in particular, promotes the development of Tregs, essential for the immune tolerance[201]. The integrity of the mucosal barrier is fundamental to keep separated the host immune cells and the gut microbiota. In fact, intestinal barrier dysfunctions have been associated with the development of IBD[202]. Antimicrobial peptides such as α-, β-, θ-defensis and cathelicidins in the small intestine are produced mostly by Paneth cells that cover a fundamental role in segregating gut Gram-positive and Gram–negative bacteria and small intestinal epithelia. At the level of the large intestine, Paneth cells are absent and the mucin glicoproteins secreted by goblet cells act as a physical barrier[203]. Here two layers of mucus, composed of globlet cell-secreted Mucin-2 (MUC2) protein, are formed. The inner mucus is anchored to the Intestinal epithelial cells (IECs), located at the surface of the mucosal barrier, and is free of gut bacteria since IECs produce defensins and transport antimicrobial IgA, preventing the penetration of microorganisms[204]. The production of these molecules by IECs is controlled by TLR4/MyD88 and NOD2 signalling that are in turns driven by gut microorganisms themselves[205,206].

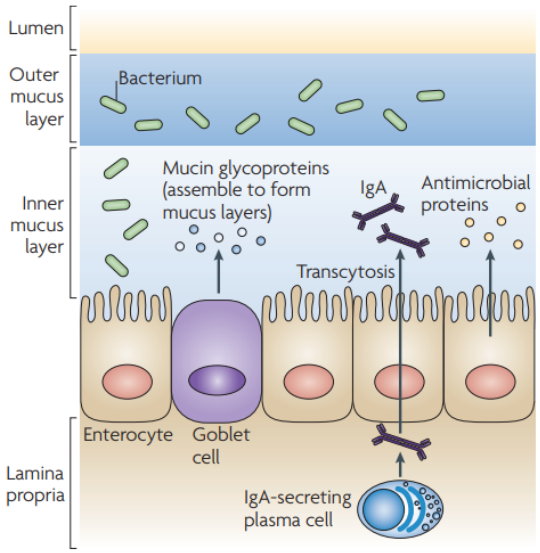


Figure 14: **Segregation of microorganisms in the lumen side of the intestinal mucosa.** Intestinal bacteria and IECs are compartmentalized in the intestine in order to maintain the homeostasis of the gut. The segregation of gut microbiota is favoured by the high sensitivity of enterocytes to the microorganisms at producing antimicrobial molecules, by the presence of a network of mucin glycoproteins and by the transport of IgAs in the mucus layer[207].

The importance of the **IgA** in the intestine is clear, as it was demonstrated to be the most abundant Ig at the mucosal site where its production is strictly tuned by the presence of commensals[208,209]. Indeed, studies performed in germ-free animals showed very low levels of IgA in these animals[210]. In the mucosal lymphoid organs PCs secrete IgA, these are bounded to the polymeric Ig receptor (pIgR) and through IECs they are transported in vesicles in the lumen after a proteolytic cleavage of the pIgR. After transport, the secretory component is left attached to the IgA: the heavy chain of two molecules of IgA are linked together by a J chain making a dimer. These Abs, under specific physiological or pathological conditions, are then spread in other mucosal compartments such as the mammary gland[211]. IgA responses are compartmentalized within the gut mucosa and, as

mentioned before, B cells that recirculate in blood and lymphoid organs preferentially home the intestine when they over-express specific receptors.

In the intestine, DCs constitutively produce retinoic acid, that induces mucosal IgA CSR in B cells and Treg differentiation (*figure 15*). Moreover intestinal B and T cells are programmed to express CCR9, which in turn directs their homing into the mucosa.

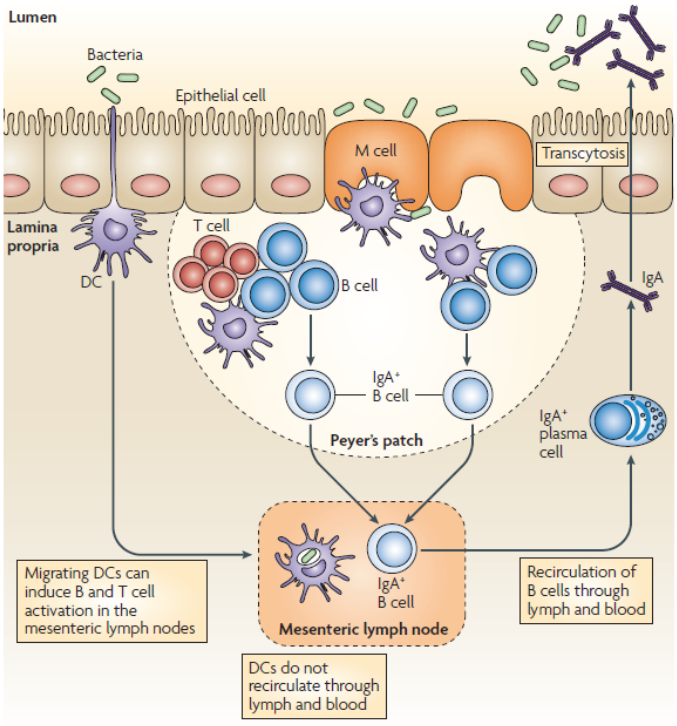


Figure 15: Intestinal DCs promote IgA PC formation. Lamina propria (LP) DCs actively sample the pathogens located at the apical surfaces of epithelial cells by extending their dendrites between the epithelial cells. Bacteria-activated DCs migrate to Peyer's patches and mesenteric LNs (MLN) where they induce B cells to differentiate into IgA^+ PCs. IgA^+ PCs in the LP produce and release dimeric IgA^+ that are transported across the epithelial layer to prevent bacteria colonization of the LP[207].

Cytokines produced by immune cells contribute in the maintenance of mucosal barrier homeostasis. Th17 lymphocytes that produce and release protective IL-17 and IL-23 in the LP are induced to develop thanks to IECs-derived serum amyloid A (SAA). The segmented filamentous commensal bacteria (SFB) instead promote the release of IL-17 and IL-22 by type3 ILCs[212]. These cytokines promote the release of anti-inflammatory peptides. When there is an impairment of the barrier, IL-4 and IL-13 Th2 cytokines promote colonic wound healing by inducing the alternative activation of macrophages and the proliferation of IECs[213].

3.3.3. Roles of MCs and B cells in diseases: focus on the intestinal environment

The protective role of differentiated B cells-derived IgA in maintaining the homeostasis of the intestine has been described above. It has also been demonstrated that IL-10 produced by B cells

has an important role in the formation of mucosal Treg cells in allergic and tumoral animal models[214,215]. MCs' contribution in tissue healing as well as in the resolution of an inflammation through the production of anti-inflammatory mediators (such as IL-4, IL-10 and TNF-β) are well known[216]. MCs, indeed, are known to be equally central in the maintenance of mucosal homeostasis in the gut since they are reported to regulate intestinal epithelial permeability during inflammatory responses[217]. In the gut MCs interact and modulate the activity of several cell types, as illustrated in *figure 16*. It has been reported, for instance, that *Kit$^{W\text{-}sh}$* or Mcpt4 K.O. mice show a decreased epithelial permeability and turnover, aside from diminished intestinal epithelial cell migration along the villus-crypt axis and altered intestinal morphology. Notably, the reconstitution of these mice with Wt MCs restored the barrier functions[218]. Moreover MC's tryptase was shown to degrade cytokines and matrix metalloproteinases (MMPs) and participates in bacterial defense[102].

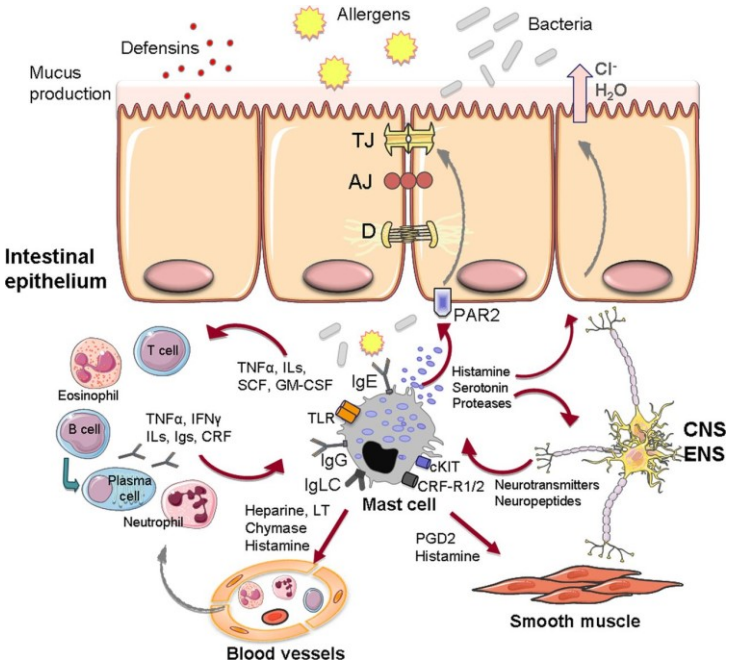


Figure 16: MC's roles in the intestinal barrier. MCs in the intestinal mucosa potentially interact with a broad spectrum of cell types. They participate in the regulation of epithelial and vascular permeability, in the recruitment and activation of other immune cells, they also modulate peristalsis and pain signalling by communicating with the nervous system[219].
AJ, adherens junction; CRFR1/2, CRF receptors 1 and 2; CNS, central nervous system; CRF, corticotropin-releasing factor; D, desmosome; ENS, enteric nervous system; GM-CSF, granulocyte and monocyte colony stimulating factor; IgLC, immunoglobulin free-light chains; ILs, interleukins; LT, leukotrienes; PAR2, proteinase-activated receptor-2; TJ, tight junction).

Since the intestine is a privileged site in which MCs and B cell reside and are shown to interact[195], we describe below how these two cell types not only are affected by, but also how they actively regulate the gut imbalanced homeostasis.

3.3.3.1. MCs and B cells in IBD

IBD encloses a group of disorders characterized by inflammation of the intestine, in human, the two major forms are Chron's disease, that can involve a wide tract of the gastrointestinal apparatus, and the ulcerative colitis (UC) that affects instead the large intestine[220]. IDB is still an incurable disease, characterized by a mutual interaction of genetic factors, intestinal epithelial barrier dysfunction, alterations in the intestinal microbiota and immune dysregulation[221]. A role of MCs in the pathophysiology of IBD has long been known from the discovery of their increased number: degranulated MCs and an increase of their mediators are reported in patients with both Crohn's disease and UC[222,223,224,225,226]. In addition, MCs have been suggested to be major drivers of acute colitis[227]. Despite an accepted role for MCs in IBD, conflicting roles of MCs are reported in different animal models. In some studies of chemical models of induced colitis, MCs are reported to be detrimental. As an example, in a rat model of intracolonic administration of trinitrobenzene sulfonic acid (TNB) and ethanol, that cause haemorrhagic and ulcerative damage to the proximal colon, the use of ketotifen, a MC's stabilizer, ameliorated the mucosal damage[228]. Moreover in dextran sodium sulfate (DSS)-induced colitis many reports showed that genetic MCs' depletion ameliorated colitis. Beyond the already mentioned importance of MC-derived MCP-4, other MC's derived mediators have been elucidated to drive the development of colitis such as the chymase, that was shown to strengthen the activity of MMP-9 in the degradation of the matrix[229,230]. However, many authors proposed that MC deficiency is associated with the exacerbation of the inflammation in spontaneous colitis models, thus proposing beneficial roles of MCs in IBD[231]. Recently, Rigoni and collaborators showed that increased MCs in the LP played a role in colonic epithelial regeneration after DSS withdrawal. They propose a model by which, MCs infiltrating the colon upon DSS withdrawal upregulate ST2, the receptor for IL-33. IL-33 is a cytokine that has been associated to intestinal inflammation and proposed as one driver of colorectal cancer (CRC): this protein and its receptors are increased in patients with IDB[232]. MCs, activated by the IL-33 release proteases such as the MCP-4, which degraded IL-33 favouring the reduction of the inflammation. Indeed, both *in vitro* and *in vivo,* MCP-4 K.O. BMMCs were unable to promote mucosal healing after DSS withdrawal, unlike Wt BMMCs reconstituted- MC-deficient mice[233]. In IL-10-deficient mice an earlier onset of spontaneous colitis and the increased intestinal permeability were observed after deletion of MCs[234]. In support of this evidence, in a double IL-10 and MCs K.O. mice model, the lack of MCs increased the pathological score and impaired the mucosal barrier, the pro-inflammatory cytokines

were increased as well as the oxidative stress and goblet cell loss[235]. It was recently confirmed the improvement of the colitis with systemic MC engraftment in these mice even if the exact mechanisms are not yet well understood[236].

Also adaptive immunity covers important roles in the pathogenesis of IBD. Considering B cells' role in IDB there are controversial reports. The association of B cells with colitis was suggested by the fact that IL-10 K.O. mice develop spontaneous colitis. Beyond the greater contribution of T cells, B cells are as well known to produce this anti-inflammatory cytokine as mentioned before. In patients suffering from UC the analysis of $CD24^{high}CD38^{high}$ and $CD5^{+}$ cells, used by the authors to identify two major Breg phenotypes, showed that the numbers were significantly decreased while an increase in the $CD95^{+}$ -exhausted Breg population was observed. Moreover, an increased serum IgG concentration was paralleled in these patients[237]. To mention, the anti-CD20 B cell depletion with the anti-CD20 rituximab, so far studied in UC, failed[238]. The inflamed intestine of IBD patients is hugely infiltrated with B cells and IgA^{+} and IgG^{+} PCs, with a skewing towards IgG production, depending on the severity of inflammation[239].

By studying the model of DSS, L. Wang and co-workers demonstrated a crucial role played by B cells in the suppression of colitis. They showed that DSS-induced colitis was more severe in B cell-lacking mice (μMT) and, contrary to previous suggestions, that IL-10 produced by B cells was not necessary for the amelioration of the inflammation. They showed instead a loop by which B cells promoted the proliferation of Treg cells in the GALT. In turns, these Treg enhanced protective IgA production. It is interesting to note that the total splenic B cell population, adoptively transferred in K.O. recipients, was responsible for the attenuation of colitis in μMT mice. This population, which accumulated in the GALT, was not IL-10 producing but was driven by Treg-derived TGF-β to switch into IgA producing PCs[240]. The importance of IgA production during colitis is also highlighted in other studies, for example IgA deficiencies predisposes to an increased rate of IBD[241,242]. In another study, IL-33 K.O. mice showed a greater body weight loss and an increased production of pro-inflammatory IL-1α; these animals had significantly decreased levels of IgA in colon explants, while the levels of other Igs were similar to Wt mice[243]. Even if the authors did not prove a direct or indirect mechanism by which IL-33 favours the production of beneficial IgA, once again they proved the importance of this mucosal Ab in the progress of the pathology.

3.3.3.2. When inflammation turns into cancer: the activation of MCs and B cells in CRC

Patients with chronic IBD, and in particular those suffering from UC, have an increased risk of developing CRC, which is the third most common tumor worldwide[244]. The association with inflammatory diseases suggests a direct interaction between immune cells in the LP and epithelial cells that undergo the adenoma to carcinoma phase [245]. Two main types of CRC can be distinguished: the hereditary colon cancer, where patients have generally loss of functions in *the adenomatous polyposis coli (APC*) gene, that leads to the destabilization of the β-catenin, and the sporadic CRC neoplasia, associated mostly to IBD.

The presence of tumor associated inflammation is an important sign of cancer progression[246] . For example MCs infiltrate the mucosa of inflamed intestine and accumulate during tumor formation (*figure 17*),suggesting a role for them in the regulation of different phases of intestinal tumorigenesis, even there are conflicting results.

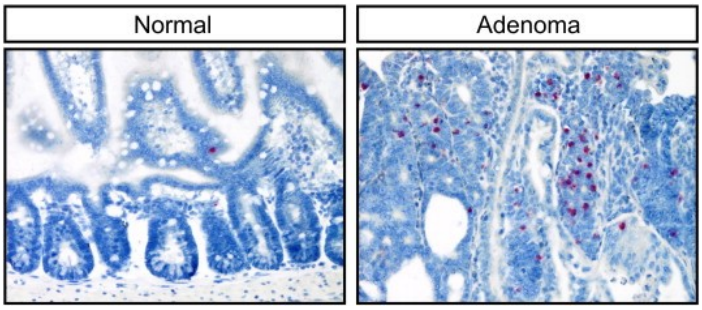


Figure 17: MCs accumulate during the progression to adenocarcinoma. A Leder stain identifies rare MCs (purple dots) in a normal mouse intestinal mucosa. MCs accumulate during cancer formation in the $Apc^{+/-}$ genetic tumor mouse model[245].

In the tumour tissue of human CRC, tryptase positive MCs have been observed to be located mainly in the stroma at the interface between rising cancer and healthy tissue and, frequently, in association with blood vessels within the tumour microenvironment (TME). The presence of MCs at the invasive margin of tumors has been associated to a poor prognosis and related to the expression of the protease-activated receptor 2 (PAR-2)[43]. One of the factors responsible for the accumulation of MCs in the tumor is the SCF produced by the tumor itself. The SCF, not only attracts MCs, but also sustains their activation to release factors (e.g. TNF-α, IL-6, VEGF, CCL2, iNOS, adenosine , MMP-9 among others) responsible to increase the vascularity, inflammation and chemotaxis of other immune populations[247] (illustrated in *figure 18*).

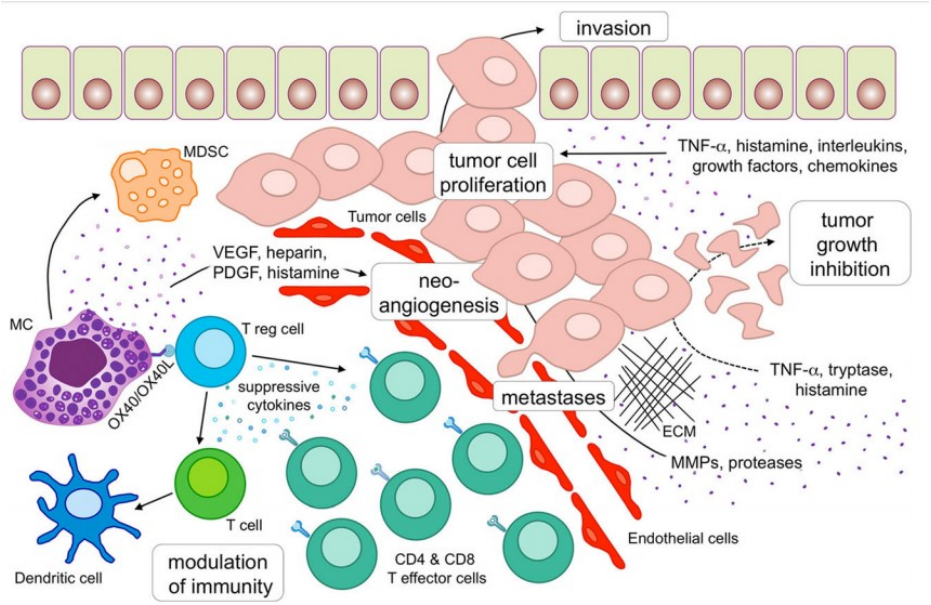


Figure 18: MCs in the TME: MCs are attracted in the TME where they release a wide range of bioactive mediators influencing the angiogenesis and the tumor growth. MCs mediators attract tumor related- immune cell subsets (such as Treg cells or myeloid derived suppressor cells (MDSC)) and cross-talk with them modulating their immune responses[248].

In CRC polyps the prognosis is often associated to a high MCs (and MC precursors) density, these cells seem indeed to promote progression cancer and sustain inflammation. MCs have been identified as an essential hematopoietic component during polyp development both in humans and in genetic APC mutated mouse models. Moreover TNF-α was identified to be an indispensable factor for MCs proliferation and expansion in $APC^{\Delta 468}$ mice produced as an autocrine factor by MCs activated in the tumor growing[249].

Direct interactions between MCs and cancer cells have been shown. For instance, MCs have been described to manifest a sort of "piecemeal degranulation" releasing factors that can promote colon cancer proliferation and invasion *in vitro* and *in vivo*. In a study, conducted in a murine setting in a Balb/c background, MCs were able to increase the expression of factors such as RhoA, VEGF, and TGF-β as well as to activate MAPK and STAT pathways in the CT26 cancer cell line[250]. In a metastatic model of CRC, MCs sustained the tumor, on the contrary a protective role of MCs has been suggested in the $Apc^{Min/+}$ mouse model in the early intestinal tumorigenesis[251]. Mehdawi and collaborators investigated in the specific the protective role of MCs in human CRC. Interestingly in their study they did not observe phenotypical differences between MCs in normal and tumor tissues but the higher MC density was associated with a longer overall survival of a cohort of patients analysed. In support to their observations, in cysteinyl leukotriene receptor 1 (CysLTR1) K.O. animals, that were previously shown to display fewer polyps, higher polyps MCs density was

observed[252]. In the previously mentioned work by Rigoni A., the authors studied also the role of MCs in CRC by using the AOM/DSS model: they showed a higher MC infiltration in most aggressive tumors[233].

In addition to TNF-α produced by MCs upon activation in the cancer context, another very important factor of the CRC TME is the IL-33. In CRC it has been shown, both in human and mice, that the epithelial cancer cells release high levels of this cytokine. In addition to the IL-33-dependent enhancement of metastatic capacity on tumor cells, it activates the stroma to create a permissive environment for tumor expansion. In 2015 the group of Heaney J.D. reported that IL-33 signalling promote polyp-associated mastocytosis by comparing normal *Apc*$^{Min/+}$ mice to *il33*-deficient *Apc*$^{Min/+}$ mice. The authors suggested that MCs establish a TME favourable to polyposis since, IL-33 activates MCs to release pro-inflammatory factors[59]. Even if there is a larger consensus concerning the pro-tumorigenic roles of the IL-33/ST2 pathway[253,254,255,256], there are some authors that showed an antitumorigenic role for ST2 in colon carcinogenesis. They detected in tumor tissue a decreased expression of the transmembrane receptor ST2L and that that IL-33 did not increase cancer cell proliferation and invasion, while the deletion of ST2 resulted in higher tumor growth *in vivo*[257].

In the analysis of lymphoid cells in the TME, tumor-infiltrating B cells (TIBs) have been considered as ignored players in tumor immunology[258]. The determination of the exact role of B cells in cancer is complicated by the fact that heterogeneous B cell subsets exist with functionally opposite roles (*figure 19*). IL-35,TGF-β and IL-10 together with PD-L1 and indoleamine 2,3-dioxygenase (IDO) expressing-B cells are important factors in immune suppression of T cells. PCs secreting IgG and IgA Abs have been instead reported to have contradictory roles. In some cases these Abs sustain the progression of cancer, but other studies show that Abs may amplify adaptive immune responses. Positive roles of B cells include the immunosurveillance facilitating the expansion and the formation of memory tumor-infiltrating T cells by acting as local APCs. Direct killing activity of B cells against the tumor can also be established. For instance, the expression of membrane death-inducing molecule Fas ligand (FasL) and the production of granzyme by B cells can directly kill tumor cells[259].

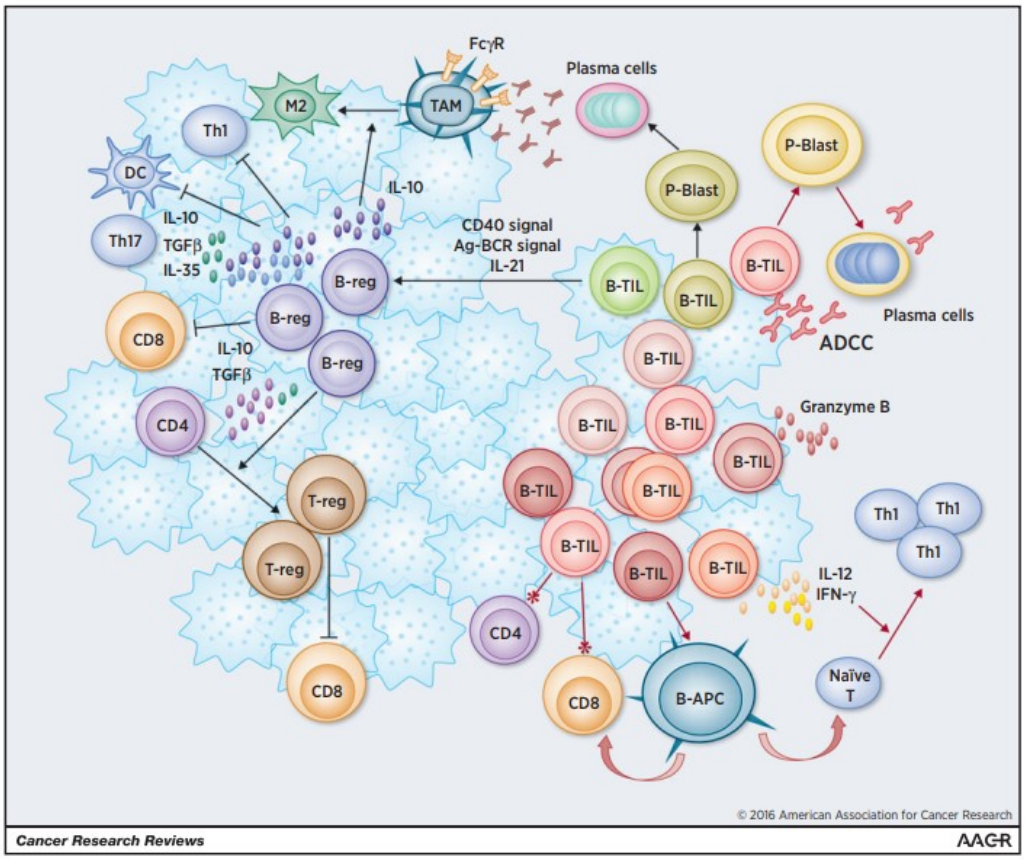


Figure 19: Different B cell subsets in the TME. Pro-tumorigenic B cell responses are played by immunosuppressive regulatory B cells that secrete IL-10, IL-35 and TGF-β as well as by IgA-expressing PCs that express IL-10, Fas-L and PD-L1. Bregs interactions with T cells induce their expression of Foxp3 and CTLA-4 (markers of Tregs). Anti-tumorigenic roles of B cells include IgG producing PCs that activate immunity against the tumor, APCs B cells for the stimulation of effector T cells, B cells expressing death-inducing molecules such as FasL (not shown in the picture) and B cells secreting granzyme B that directly induce apoptosis on cancer cells[260].

In CRC, infiltrating B cells were described at the invasive margin in lymphoid aggregates in close contact with DCs, this structure is known as “Crohn's like reaction”. The B cell populations identified were composed of mature B cells and of differentiated PCs. Moreover, high levels of MHC-I and -II and CD80 were detected on these TIBs. Ag-experienced PCs secrete Ig of the three types IgG, IgA, IgM with predominant polarization towards IgG[261]. Later, in a work conducted on human patients aimed at characterizing B cells in CRC, a higher percentage of memory B cells ($IgD^{-}CD27^{+}$) was identified in peripheral blood compared to healthy controls. Moreover, there was a reduced number of B cells in liver metastasis acquiring a regulatory phenotype. On the contrary, early-stage cancers were enriched in terminally differentiated PCs both in the blood and the tumor suggesting an antitumor response[262].

In our laboratory, we recently published a work in which we performed a fine characterization of B cell phenotypes and functions of B cells in lymphoid organs of murine models of CRC with a

particular focus in the genetic model of the *Apc*$^{Min/-}$ mouse. We observed that IL-10 competent B cells were increased in the LNs draining the tumor (dLNs) and that in the spleen and peritoneum there was an increased proportion of IgA^{+} lymphocytes. The increase of IgA PCs was also confirmed in the analysis of serum IgA Abs and in the identification of IgA^{+} cells in the transformed gut mucosa. Interestingly, we observed that the percentages of B cells in both MLNs of the *Apc*$^{Min/-}$ mouse model and AOM/DSS tumor model and in the dLNs of the subcutaneous model of ectopic CRC in CT26-bearing mice were increased. Preliminary evidences suggest that this B cell accumulation was due to a higher expression of physiologic and inflammatory of lymphocytes-related chemokine: CXCL12, CXCL13, CCL19, CCL21 and CCL20[263]. This microenvironment favourable to a B cell recruitment at the level of tumor growth is extended in the context of this thesis work where a new interesting MC-tumor cell-B cell axis is proposed.

Increased chemokines are indeed observed in several cancer types and are responsible for the infiltration of tumor leukocytes and for the process of metastatization of solid cancer such as the CRC. For instance, CCL2 and CCL5 have been correlated with high levels of tumor associated macrophages (TAMs) that promote angiogenesis and inhibit T cell responses in breast cancer[264,265]. CCR7 expression was reported to be crucial in melanoma, CXCL12 was seen to be involved in metastatization in the lungs and has been proposed as a therapeutic target[266,267].

In CRC many chemokine-receptor couples have been identified in cancer progression and metastasis, these axis are listed in the *table3* and *picture 20*.

In this thesis, the CXCR6-CCL20 axis will acquire a novel perspective with regards to the attraction of B lymphocytes in CRC tumor sites. From the literature it is known that increased levels of CCL20 and CCR6 have been first described in human samples of primary and metastatic CRC compared to normal mucosa[268,269]. CCL20 is weakly expressed in normal colonic mucosa but in response to an inflammatory stimulus CCL20 is strongly up-regulated. The higher expression of CCR6 on CRC liver metastases suggested the hypothesis of a role played by CCR6 in the metastatization process[270]. CRC cells express in fact both CCL20 and CCR6 in a non-polarized manner. In this way efficient autocrine and paracrine loops are established[271]. These evidences have also been confirmed in animal models. Interestingly, in the generated CCR6 K.O.-*Apc*$^{Min/+}$ mice, fewer intestinal tumors and smaller spleens (spleens size correlates with burden of intestinal adenoma) were developed compared to the *Apc*$^{Min/+}$ mice[272]. In the same work the authors demonstrated a role for this axis for both macrophage infiltration and CCL20-induced proliferation of murine and human CRC cell lines through an auto-feedback loop. In another work it has been shown that TAMs are an

importance source of CCL20 in the tumor progression[273]. To date, the main cell types clearly reported to be recruited through this axis in CRC beyond monocytes are Th17 and Tregs [274,275].

Table 3: Chemokine axis proposed to be central in CRC progression[275]

Chemokine Signaling	Expressing Cell Type		Function
	Ligand	Receptor	
CXCL1–CXCR2	CRC	EC *	Angiogenesis
	Liver	CRC	Liver metastasis
	Inflamed colon	MDSC	Colitis-associated tumorigenesis
CXCL9/10–CXCR3	Lymph node	CRC	Lymph node metastasis
	CRC	Th1	Anti-tumor immunity
CXCL12–CXCR4	Liver	CRC	Liver metastasis
	EC *, myofibroblast	CRC	Liver metastasis
	CRC	ND †	Shorten OS and RFS
CCL2–CCR2	CRC	TAM	Disease progression
	CRC	BMDC	Liver metastasis
	CRC	MDSC	Carcinogenesis
	CRC	EC, monocyte	Extravasation and metastasis
CCL5–CCR5	CTL	CTL, Th1	Anti-tumor immunity
	CRC	T-reg	Tumor growth
CCL15-CCR1	CRC	BMDC	Invasion and liver metastasis
	CRC	TAN	Liver metastasis
	CRC	MDSC	Invasion
CCL20–CCR6	TAM	T-reg, Th17	Tumor progression
CCL19–CCR7	DC ¶	CTL	Anti-tumor immunity
CX3CL1–CX3CR1	ND †	TAM	Liver metastasis

* EC: endothelial cell; ¶ DC: dendritic cells; † ND: not described.

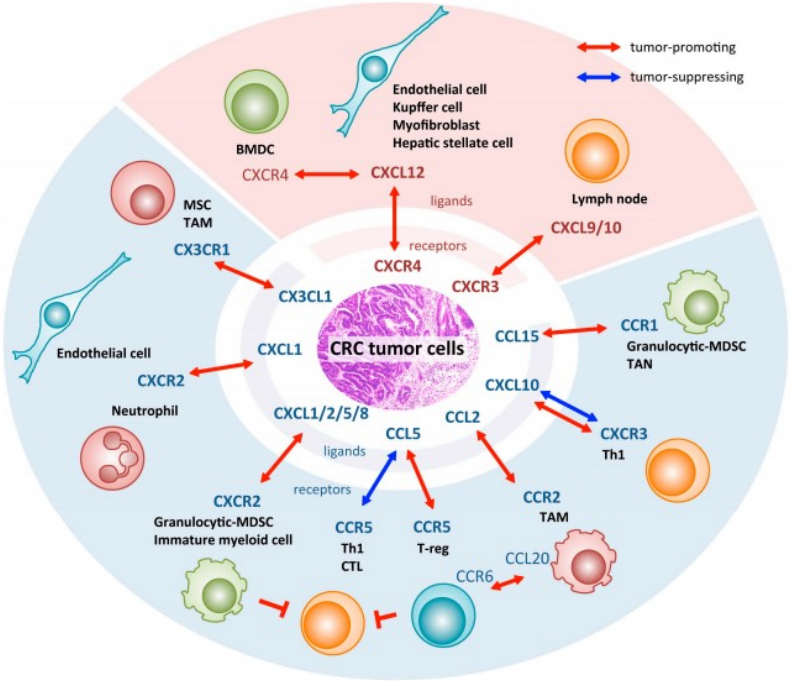


Figure 20: CRC cells-leukocytes interactions mediated by chemokines. In the TME chemokine-receptor signallings are established between tumor cells and leukocytes. Tumor-promotive (red arrows) and tumor-suppressive (blue arrows) effects are produced following the recruitment of myeloid and lymphoid cell populations (BMDC: bone marrow derived cells)[275].

4. AIMS OF THE THESIS

MCs have increasingly gained notoriety for their multiple roles, not only in allergies, but also in pathological contexts such as acute inflammation and cancer. Their multiple phenotypes and functions and the continuous cross-talking with other immune cell types are among the most fascinating aspects in MCs' biology. In previous works our laboratory uncovered an interesting cross-talk, at the level of normal and inflamed intestine, established between MCs and B lymphocytes: MCs were shown to be capable of regulating and supporting several aspects of conventional B cells' functions. The purpose of this thesis work was to investigate some unaddressed aspects of MC/B cell cross-talk in physiological and pathological contexts. Since sub-populations of both MCs and B cells can interact in specific and separate body compartments, the first line of research aimed to study the interaction between different phenotypical and functional MCs subtypes with the B-2 and B-1 lineages of B lymphocytes. Since in many B cell-related pathologies increased numbers of MCs are reported, beyond the evaluation of the effects that MCs induce on B cell subtypes, we were interested in clarifying whether B cells could reciprocally favour or enhance the activation of MCs. After an *in vitro* study, some of the main observations were investigated also in mouse models. The distribution of MCs was analysed in the B-1 lacking *bumble* mice because of the interesting data emerged in the MCs/B-1 cells interaction. A systematic analysis of the distribution and activation of B cells was performed in two MCs-deficient models where both the homeostatic and the DSS-induced colitis contexts were examined because of the great importance of the MC/B cell cross-talk in the intestine.

As a second aim, the MC/B cell interplay was investigated in the CRC since this pathology can be a detrimental consequence of a persistent inflammatory state of the gut. Our recent data showed that the onset of CRC dramatically affects the distribution and phenotype of B lymphocytes and it is known that MCs are important immune drivers of this cancer type. The MC/B cell interaction was explored taking advantage of an *in vivo* subcutaneous model of CRC in Wt and MC-deficient animals. The analysis of the direct cross-talk between MCs and cancer cells helped us to determine how MC's activation in this cancer context was responsible for some alterations of the B cell behaviour in this pathology.

5. RESULTS

5.1. MC-B CELL BIDIRECTIONAL CROSS-TALK UNDER PHYSIOLOGICAL CONDITIONS: THE MC SIDE

The latest advancements in MC's biology show the great heterogeneity in phenotypes and functions acquired by MCs under the pressure of different physiological and pathological microenvironments[5]. The different body compartments are determined by the specificity of resident cell subsets and of their mediators and B lymphocytes and are one relevant cell type that populates many MCs' sites of differentiation. With the aim of extending the knowledge about MCs and B cells interplay, we decided to investigate some of the unanswered traits of the MC-B cell cross-talk in the murine setting. MC's support in conventional B-2 cells-related functions is widely accepted: activation and isotype switching are favoured in the presence of soluble mediators released by MCs and by effect of a membrane-dependent cross-talk[195,198]; moreover, MCs induce the expansion and differentiation of IL-10-competent B cells[196]. On the contrary very little is known on the effects that B cells can have on MCs' activation even if the concomitant increase and activation of MCs in many B cell-related inflammatory and tumoral pathologies suggest that, *in vivo*, B cells may affect MCs behaviour[194,276]. Moreover, some of the *in vivo* sites in which MCs are located and in which their activation is of great relevance in pathological conditions such as the peritoneal cavity and the intestine[277,94] are populated to a great extent by innate-like B-1 cells. As a first objective we explored the effects that B cells can induce on the biology of the two main subtypes of murine MCs exploring in particular the modulation of activator membrane-bound receptors and the ability to release regulatory and inflammatory mediators, both in resting conditions and during an IgE/Ag dependent-stimulation. The second objective of the *in vitro* characterization of the mutual MC-B cell interplay was focused on defining common and different effects induced by MCs in the biology of B cell subsets. To address these aims we established an experimental set up (described in the methods section, *paragraph 7.2.8*) in which *in vitro* bone marrow-derived MCs (BMMCs) that are immature mucosal-like MCs and mature serosal-type peritoneal-derived MCs (PDMCs) were co-cultured with freshly purified murine splenic or peritoneal cavity B cells. These two different anatomical B cell populations are representative of B-2 conventional lymphocytes or enriched in B-1 innate-like lymphocytes respectively.

5.1.1. B cells do not affect MCs degranulation

The degranulation of the MC in an allergic context is the best known sign of its activation and it is the result of the antigenic stimulation through the cross-linking of IgE bound at its high affinity receptor FcεRI[278]. In literature it has been shown that conventional T cells are able to potentiate MC's degranulation and the synthesis of mediators through the physical contact and the release of soluble vesicles[279]. Treg cells induce the suppression of MC's allergic activation through the OX40-OX40L axis[280]. However, through a TGF-β dependent mechanism, Treg are also reported to enhance IL-6 production in MCs[281]. Concerning B cells' effect on MCs degranulation, in 2016 Palm and collaborators reported that resting and BCR-activated splenic B cells did not modulate MCs' degranulation[198]. Similarly to what observed with Treg cells, IL-10-producing $CD5^+$ splenic B cells have been shown to reduce FcεRI signalling in MCs by decreasing Syk activation which results in a diminution of degranulation[197].

From these evidences, it is clear that there is no consensus on B cell's role on MC's degranulation and moreover this seems to be different depending on the specific B cell subset analysed. We decided therefore to deeper investigate the effect of B cells on MC's degranulation by comparing the effect given by conventional splenic B-2 and peritoneal B-1 cells. IgE pre-sensitized BMMCs were challenged in Tyrode's buffer with DNP for 30 min in the presence or absence of an equal amount of splenic or peritoneal cavity B cells. To detect basal levels of degranulation also unstimulated BMMCs were put in the same buffer solution for 30 min in the presence of B cells. Degranulation extent was measured in terms of the amounts of β-hexosaminidase, an enzyme enriched in MC's cytoplasmic granules, released after 30 min of DNP challenge as described in the experimental procedures *paragraph 7.2.3*. Our data show that both the basal release of β-hexosaminidase and the degranulation of MCs induced by a high Ag concentration (50 ng/ml) was only minimally increased over the control by both the B cell subsets analysed (*figure 21A*). Statistically significant increases are indicated, however from a biological point of view these differences can be considered marginally relevant. We also tried to boost B cells' effect by pre-stimulating the cells with LPS for 48h before culturing them with BMMCs. However, even when activated, no biologically relevant effects were observed (right graph *panel A*). We then hypothesized that B cells support could have a role in the presence of lower doses of Ag. Therefore, the same experiments were repeated activating BMMCs with intermediate (10 ng/ml DNP) and low Ag concentrations (1 ng/ml DNP). However our results showed that, even in the presence of lower Ag concentrations, B cells are not

able to promote a biologically relevant increase of MC's degranulation. Moreover, the representative experiment conducted in the analysis of Leukotrienes C4, D4 and E4 support this observation since the levels of leukotrienes were unchanged in the presence of B cells (*figure 21C*). Altogether, these data indicate that, at least *in vitro*, MC's anaphylactic degranulation is not potentiated neither by the B-1 nor the B-2 B cell subsets and this distinguishes the MC-B cell interaction from the cross-talk with T cells.

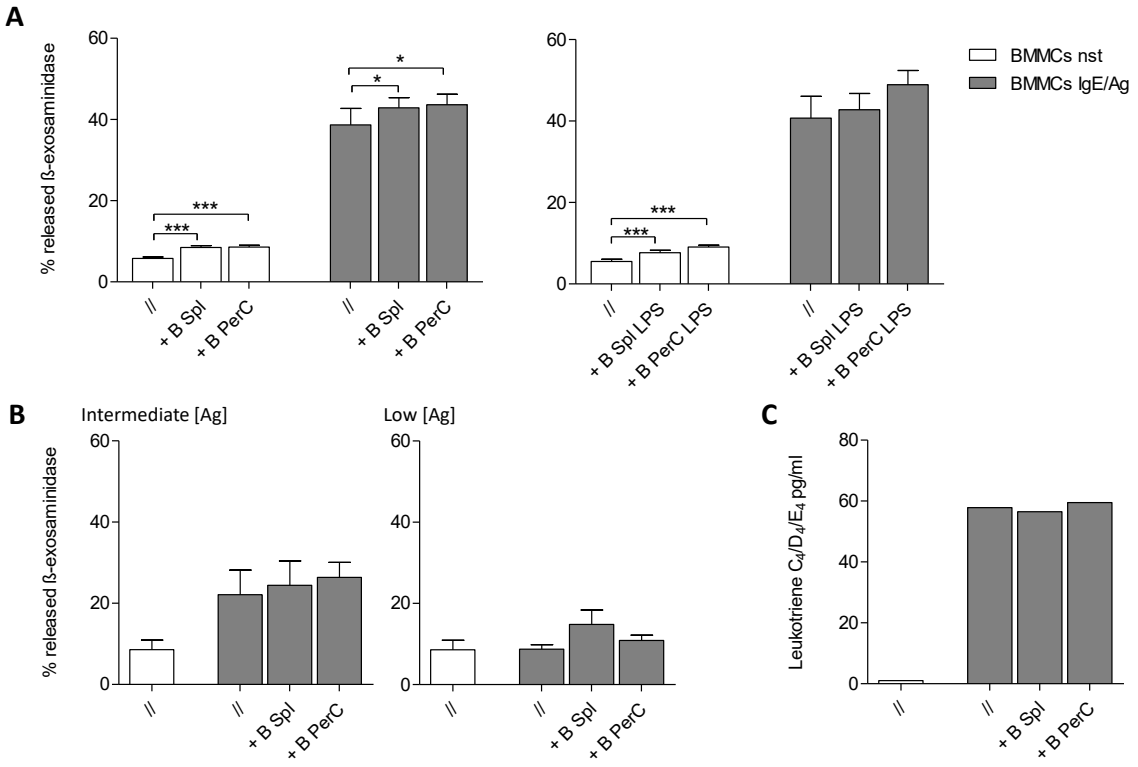


Figure 21: The degranulation of MCs is slightly affected by the presence of both B-2 and B-1 enriched B cell populations. (A) β-hexosaminidase release by not stimulated (nst) or anti-DNP IgE pre-sensitized BMMCs challenged with 50 ng/ml of DNP after 30 min from the addition of unstimulated splenic (Spl) or peritoneal cavity (PerC) B cells (left panel) or 48h 1µg/ml LPS-stimulated B cells (right panel). (B) 10 ng/ml or 1 ng/ml (respectively left and right panel) of DNP are used to induce BMMCs' degranulation under intermediate and low Ag concentrations respectively. (C) Detection of the release of leukotrienes from activated BMMCs cultured with freshly purified B cells under high Ag concentration (50ng/ml DNP). A representative experiment is shown. (A, B) Means (+SEM) from at least three independent experiments are shown. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ by one-way ANOVA done on nst and IgE/Ag conditions.

5.1.2. Peritoneal cavity B cells modulate the expression of MCs' membrane-bound molecules

MCs express a considerable amount of activating and inhibitory receptors that can fine tune the level of MCs' activation[282]. In this light, we decided to explore the possibility that B cells could modulate the expression of some membrane-bound molecules on MCs. We decided to compare the effect of splenic and peritoneal cavity B cells on BMMCs and PDMCs in terms of ICAM-1, CD28, MHC-II, ST2, LAMP-1 and c-Kit expression.

Table 4: Membrane-bound molecules of MCs analysed in the co-culture system

Molecule	Level of expression	Functions	References
ICAM-1 (intercellular adhesion molecule-1)	Constitutive	Up-regulated in response to inflammatory stimuli. IL-33 induces ICAM-1 up-regulation by inducing the NF-kB signalling	[283]
CD28	Low levels	Co-stimulatory molecule. Modulated by bacteria products. When engaged together with the FcεRI, it increases the release of TNF-α by MCs	[284]
MHC-II	Very low levels/ inducible	Present to a limited level at the cell surface and mainly in intracellular granules. Expression increased following stimulations with stimuli such as LPS, INF-γ or IL-33.	[285,286,287]
ST2	Highly expressed	Receptor of the IL-33 cytokine. Among the markers for the phenotyping.	[288]
LAMP-1 (late phase marker lysosomal-associated membrane protein 1) or **CD107a**	Induced upon degranulation	Marker used to follow MC degranulation by flow cytometry.	[289]
c-kit	High levels	Main marker that through the binding of the SCF is able to influence virtually all the MC's biologic activities	[290]

In accordance with what reported in literature we first observed that, under resting conditions, only c-kit and ST2 were more expressed in PDMCs compared to BMMCs (respectively a fold induction of 1.3 and 1.5), while all the other molecules were expressed at comparable levels (*figure 22*) [291]. In the presence of B cells, no modulations of ICAM-1, CD28 and LAMP-1 expression were observed on resting and activated MCs. Of note, the result that B cells did not affect the expression of LAMP-1 after 1h of IgE/Ag activation of MCs confirms the evidences of the β-exosaminidase dosage experiments and, therefore, the conclusion that B cells do not provide any support in MCs'

degranulation. On the other hand, the expression levels of MHC-II, ST2 and c-Kit underwent modulation in the presence of B cells. More in detail, in the presence of both B cells' subsets, MHC-II expression increased on both resting and activated PDMCs and BMMCs, suggesting that B cells can promote the reported MCs' inducible activity to process and present antigens to CD4+ T cells[286]. Regarding ST2, its expression was further upregulated both on BMMCs and PDMCs in the presence of peritoneal cavity, but not splenic, B cells. Therefore, the MC/peritoneal cavity B cell interplay is able to promote the increase of an "inflammatory" receptor, which can lower the threshold of MCs activation in the presence of tissue injury, which results in IL-33 release. It is particularly relevant that IL-33 binding to ST2 is actually able to induce the release of pro-inflammatory mediators from MCs and to potentiate the IgE-dependent activation, therefore sustaining inflammation[55]. Finally, we observed a downregulation of c-Kit in IgE/Ag activated PDMCs, also in this case given by peritoneal cavity B cells.

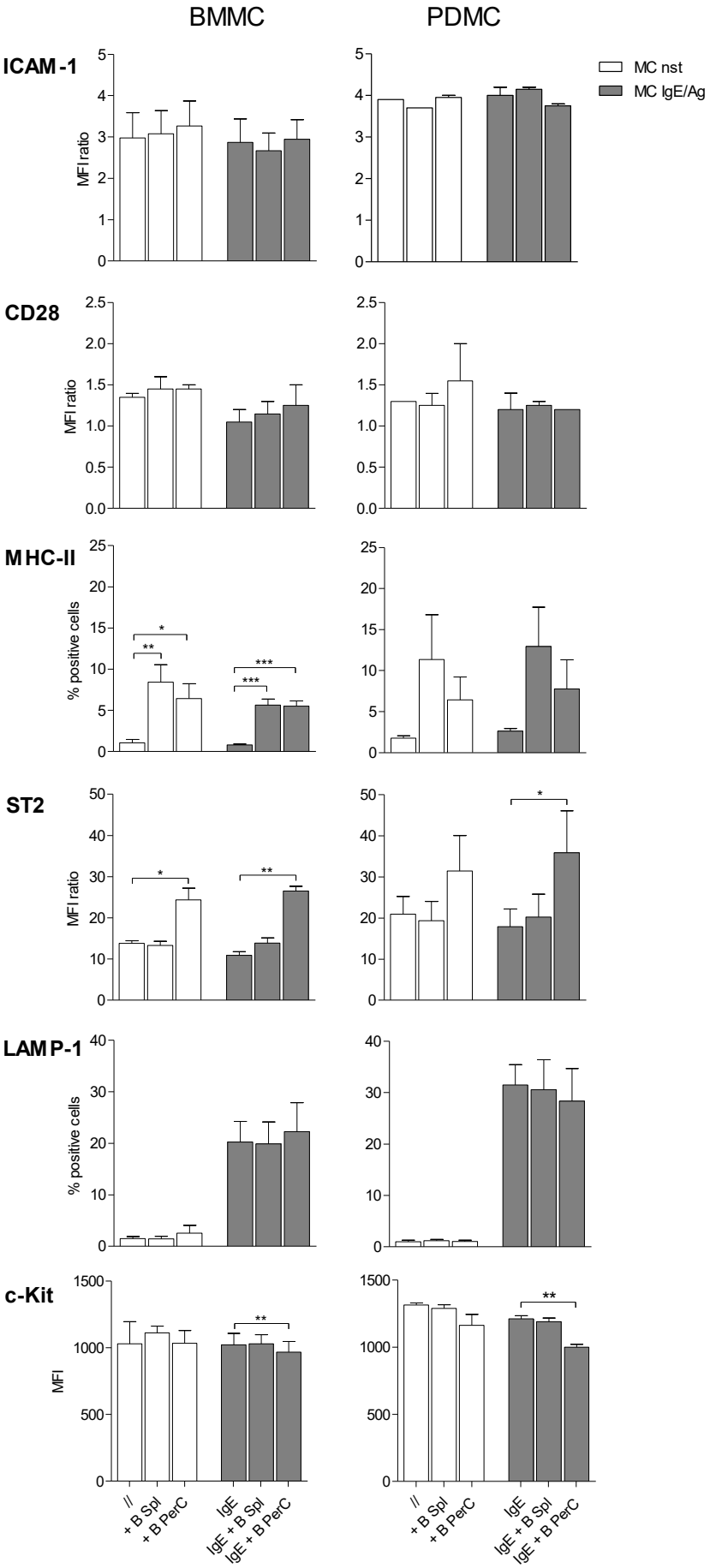


Figure 22: MCs' expression of MHC-II, ST2 and c-Kit are differently modulated in the presence of splenic and peritoneal cavity B cell subsets. Resting (nst) or 100 ng/ml DNP challenged IgE stimulated MCs (IgE/Ag) were co-cultured with splenic (Spl) or peritoneal cavity (PerC) B cells. Following co-culture, c-Kit$^+$ MCs were analysed by flow cytometry for the expression of specific membrane molecules. Both BMMCs (left column) and PDMCs (right column) were analysed. ICAM-1, ST2 and c-Kit were analysed after 24h of co-culture, MHC-II and CD28 at 48h, while LAMP-1, a MC's marker of degranulation, was evaluated after 1h of co-culture. Expression levels of c-Kit is indicated as mean fluorescence intensity (MFI), ICAM-1, CD28 and ST2 are indicated as MFI over the control isotype (MFI ratio). MHC-II and LAMP-1 molecules, that are not uniformly expressed by the MCs populations, are expressed as percentage of positive cells in the total population analysed (% positive cells). Means (+SEM) from at least three independent experiments are shown. *p<0.05, ** p<0.01, *** p<0.001 by one-way ANOVA done on nst and IgE/Ag conditions.

5.1.3. Activated MCs-related soluble mediators are increased in the co-culture system

Beyond the expression of a large number of membrane-bound receptors and co-stimulatory molecules, MCs can release a wide range of *de novo* synthesized mediators that are important in the modulation of immune responses to pathogens, and to orchestrate the behaviour of other immune and non-immune cells[292].

We observed that B cells, and in particular B-1 enriched peritoneal B lymphocytes, were able to modulate MC's expression of membrane-bound molecules (MHC-II, ST1 and c-Kit), but had no effect on MCs' degranulation. Therefore, as a next step we decided to assess whether B cells could affect the ability of MCs to produce and release soluble mediators. In particular, we chose to focus on soluble mediators which are important in the contexts of initiation and resolution of an inflammatory process. We established co-cultures between BMMCs and splenic or peritoneal cavity B cells and, at different time points, supernatants were collected and tested for the soluble mediators listed in *table 5*. As reported in *table 5*, the cytokines IL-1β, IL-4, IL-5 and IL-33 were not detectable in our system while there was no modulation of the release of the anti-inflammatory cytokine IL-10 (*figure 23*).

Table 5: Soluble mediators analysed in the MC-B cell co-culture system. The presence of different soluble mediators was assessed in the supernatants collected at different time points in the MC-B cell co-culture system. In our conditions, measurable levels of IL-6, IL-10, IL-13, TNF-α and CCL2 were detected.

Mediator	Detectable in the system
IL-1β	no
IL-4	no
IL-5	no
IL-6	*yes*
IL-10	*yes*
IL-13	*yes*
IL-33	no
TNF-α	*yes*
CCL2	*yes*

Interestingly, the amount of IL-13, an important mediator for allergic inflammation in several tissues[293] released by MCs after IgE/Ag activation was two-fold higher in the presence of peritoneal B cells. A very similar result was obtained for the pro-inflammatory cytokines TNF-α, IL-6 and CCL2. Also in these cases, the release was enhanced only by peritoneal cavity and not splenic B cells. As

observed for the modulation of MC's surface molecules, these results suggest that a specific cross-talk is established between MCs and peritoneal cavity B cells.

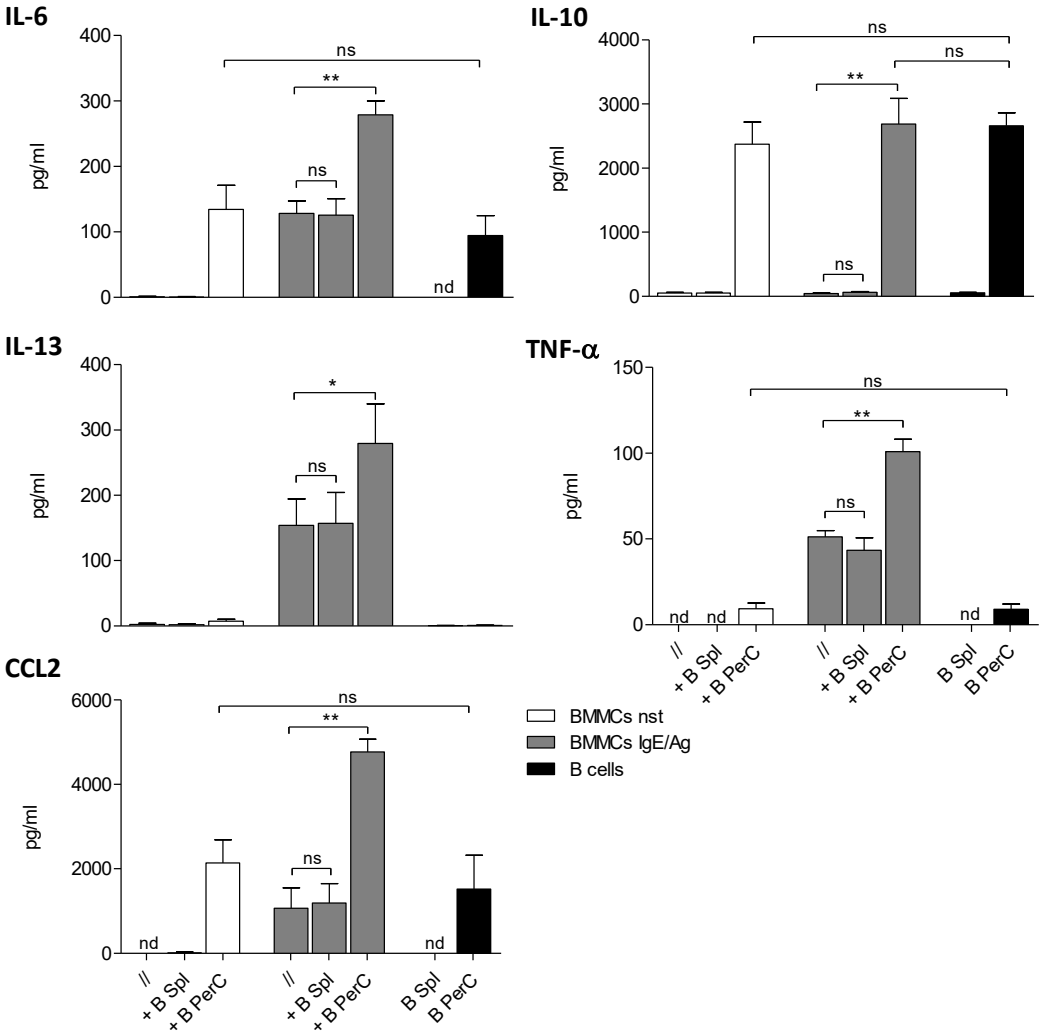


Figure 23: Analysis of the release of mediators in the co-culture between BMMCs and splenic *versus* peritoneal cavity B cells. Secreted IL-6, IL-10, IL-13, TNF-α and CCL2 from resting (nst) and activated BMMCs (IgE/Ag) alone (//) or cultured for 48h (IL-6, IL-10 and IL-13), 24h (TNF-α) or 6h (CCL2) with splenic or peritoneal cavity B cells were detected by ELISA. The B cells alone condition was also evaluated. The results showed are average (+SEM) of values from at least three independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ by one-way ANOVA. nd=not detected; ns=not significant.

5.1.4. B-1 B cells support the neo-synthesis of the pro-inflammatory mediators TNF-α and CCL2 in activated MCs

Except for IL-13, all the other mediators tested were produced by both MCs and B-1 cells (*figure 23*). Therefore, further investigations were needed to determine which of the two cell types was

responsible of the increased amount of soluble mediators detected in the co-culture supernatant. Given the importance of TNF-α and CCL2 in MC's biology, we decided to focus on these two soluble mediators.

MCs are an important cellular source of TNF-α which can be released as a consequence of two different processes. Indeed, MCs can store granules of TNF-α that are immediately released during degranulation but they are also able to synthesize new TNF-α proteins after an IgE/Ag-dependent stimulation and therefore release TNF-α within longer time points (e.g. 24h)[69]. To better characterize the modulation of TNF-α release, we first repeated the co-culture experiment using PDMCs in place of BMMCs, to determine if the observed data were the result of a specific cross-talk established between peritoneal B cells and mucosal-type MCs or a general mechanism observed with different MC subtypes. As shown in *figure 24*, a higher release of TNF-α was observed also from IgE/Ag stimulated PDMCs. *In vivo* the high importance of TNF-α derived from MCs in the peritoneal cavity during infections has long been known[294] and in our experiments, a stronger increase of TNF-α release has been observed by co-culturing the two peritoneal cavity cell types (*figure 24*).

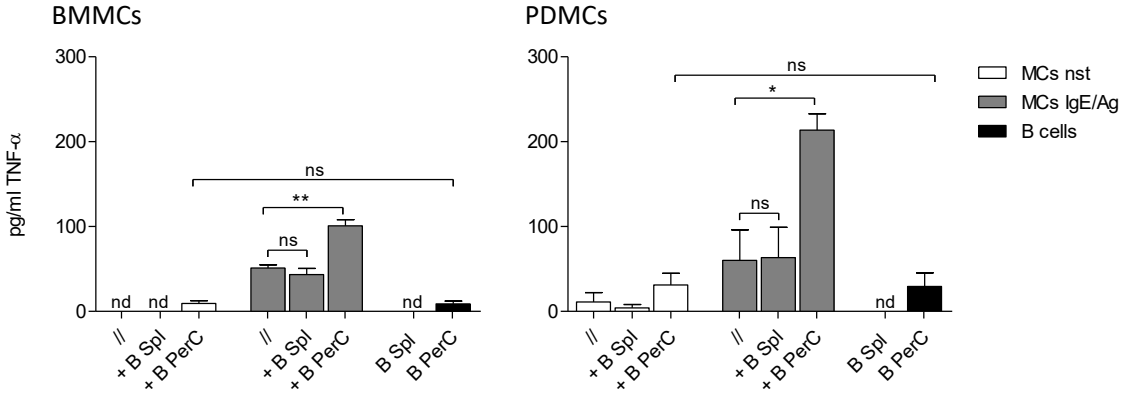


Figure 24: Comparison of TNF-α release in co-cultures between B cells and BMMCs or PDMCs. Secreted TNF-α from resting (nst) and activated (IgE/Ag) BMMCs (on the left) or PDMCs (on the right) alone or cultured for 24h with splenic or peritoneal cavity B cells is detected by ELISA. Reported results are mean (+SEM) of values from at least three independent experiments. *p<0.05, ** p<0.01 by one-way ANOVA. ns=not significant; nd=not detected.

We then performed a time course analysis after 30min, 1, 6, and 24h of co-culture between BMMCs and peritoneal cavity B cells to determine the kinetic of the TNF-α release. Although at 30 min we did not observe any relevant TNF-α release (probably due to the sensitivity of the ELISA kit), at 1h we were able to detect a statistically significant increase of the amount of TNF-α which reached its maximum after 6h of co-culture (*figure 25A*). After having more deeply established the importance

of the B PerC/MC crosstalk in the production of TNF-α, we finally performed the co-culture experiment at 1 and 6h using Wt and TNF-α K.O. BMMCs (kindly provided by the group of Prof. Kollias G, Vari) in order to determine the cellular source of this mediator. As shown in *figure 25B,* our results show that MCs were the major contributor of TNF-α production. In fact, in the case of TNF-α K.O. BMMCs, the concentration of TNF-α in the co-culture was even lower than that detected for peritoneal B cells cultured alone (*figure 25*).

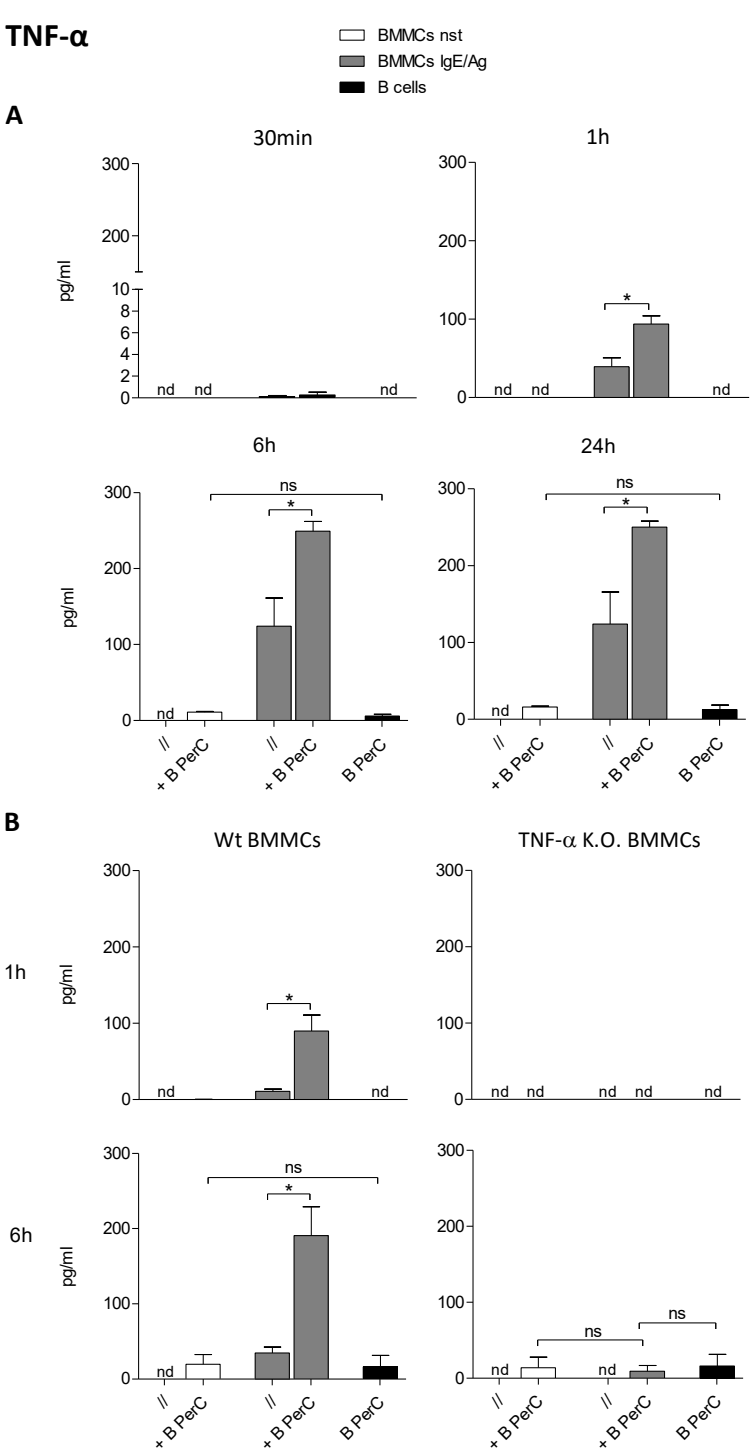


Figure 25: The analysis of TNF-α release in the BMMCs-peritoneal cavity B cells co-culture revealed an early effect induced by peritoneal cavity B cells on activated BMMCs. (A) Time course analysis of released TNF-α from resting (nst) and activated BMMCs (IgE/Ag) alone or cultured for 30 min, 1, 6 and 24h with peritoneal cavity (PerC) B cells. **(B)** The same co-culture with PerC B cells has been established with Wt and TNF-α K.O. BMMCs and supernatants were analysed by ELISA for soluble TNF-α. Means (+SEM) from at least three independent experiments are shown. *p<0.05 by one-way ANOVA. nd=not detectable. ns=not significant

In order to understand whether peritoneal cavity B cells are acting on the pre-formed or on the newly synthesized TNF-α, we pre-treated BMMCs with Brefeldin A (BFA) and performed an intracellular cytokine staining (ICS) for TNF-α after 1h of co-culture. Indeed, after IgE/Ag stimulation, the TNF-α pre-stored in the granules is released in the culture media while the newly synthesized TNF-α is trapped in the cytoplasm and can be detected through ICS (the gating strategy in reported in *figure 26A*). As shown in *figure 26B-C*, in the presence of an enriched B-1 population and during

an IgE/Ag stimulation, a neo synthesis of the pro-inflammatory mediator is induced since the percentage of TNF-α⁺ BMMCs increases from 25% to 45%. We concluded that a potentiation of the protein synthesis was reflected in a stronger release of the mediator.

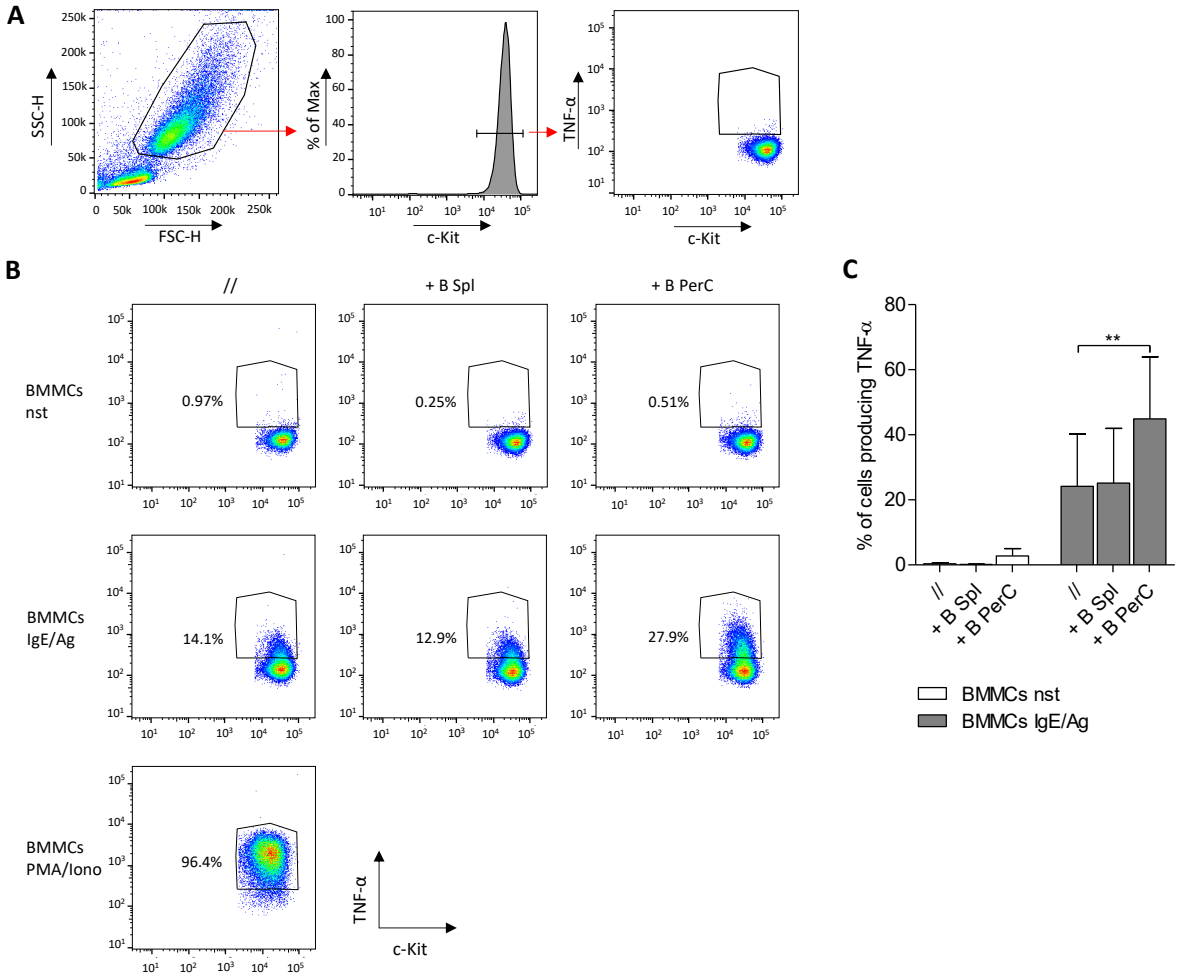


Figure 26: Peritoneal cavity B cells induce an increase of TNF-α production by MCs. ICS for TNF-α performed after 1h of co-culture between BMMCs and splenic or peritoneal B cells. **(A)** Gating strategy used for the flow cytometry analysis. BMMCs, selected on the basis of their morphology and of c-Kit expression, were then analysed for the positivity of TNF-α. **(B)** A representative experiment shows all the conditions analysed. Columns indicate whether BMMCs were cultured alone (//) or in the presence of splenic (Spl) or peritoneal cavity (PerC) B cells, rows show unstimulated (nst) or activated (IgE/Ag) MCs. The stimulation with PMA and ionomycin (PMA/Iono) is shown as a positive control since it is known to have a strong effect on TNF-α neo-synthesis. **(C)** In the graph, the mean percentages (+SEM) of TNF-α producing cells from three independent experiments are indicated. **p<0.01 by one-way ANOVA.

Moving to CCL2, in order to assess whether the increase of CCL2 shown in *figure 23* originates from MCs, we took advantage of CCL2-eGFP (green fluorescent protein) K.I. mice (generated in the laboratory of Prof. Blank U. in Paris) in which the production of the CCL2 protein is coupled with the one of the GFP protein. More specifically, CCL2 e-GFP BMMCs were first treated with BFA to allow

the accumulation of the fusion protein and its detection by flow cytometry. Then, we set up the co-cultures with peritoneal cavity B cells and performed a two points time course analysis (1 and 5h). As shown in panel A of *figure 27*, we were able to observe a positive shift in the fluorescence on activated BMMCs after 5h of co-culture with B-1 enriched B cells. This result was confirmed analysing the co-culture through fluorescence microscopy. Indeed, the representative images reported in *figure 27C* show an increase of the granularity of MCs and of the green fluorescence in the intracellular compartment of MCs that were cultured for 5h in the presence of peritoneal B cells compared to the resting condition. Therefore, the results obtained from the analysis of CCL2 production, lead us to speculate that peritoneal cavity B cells promote the production of pro-inflammatory factors by activated MCs.

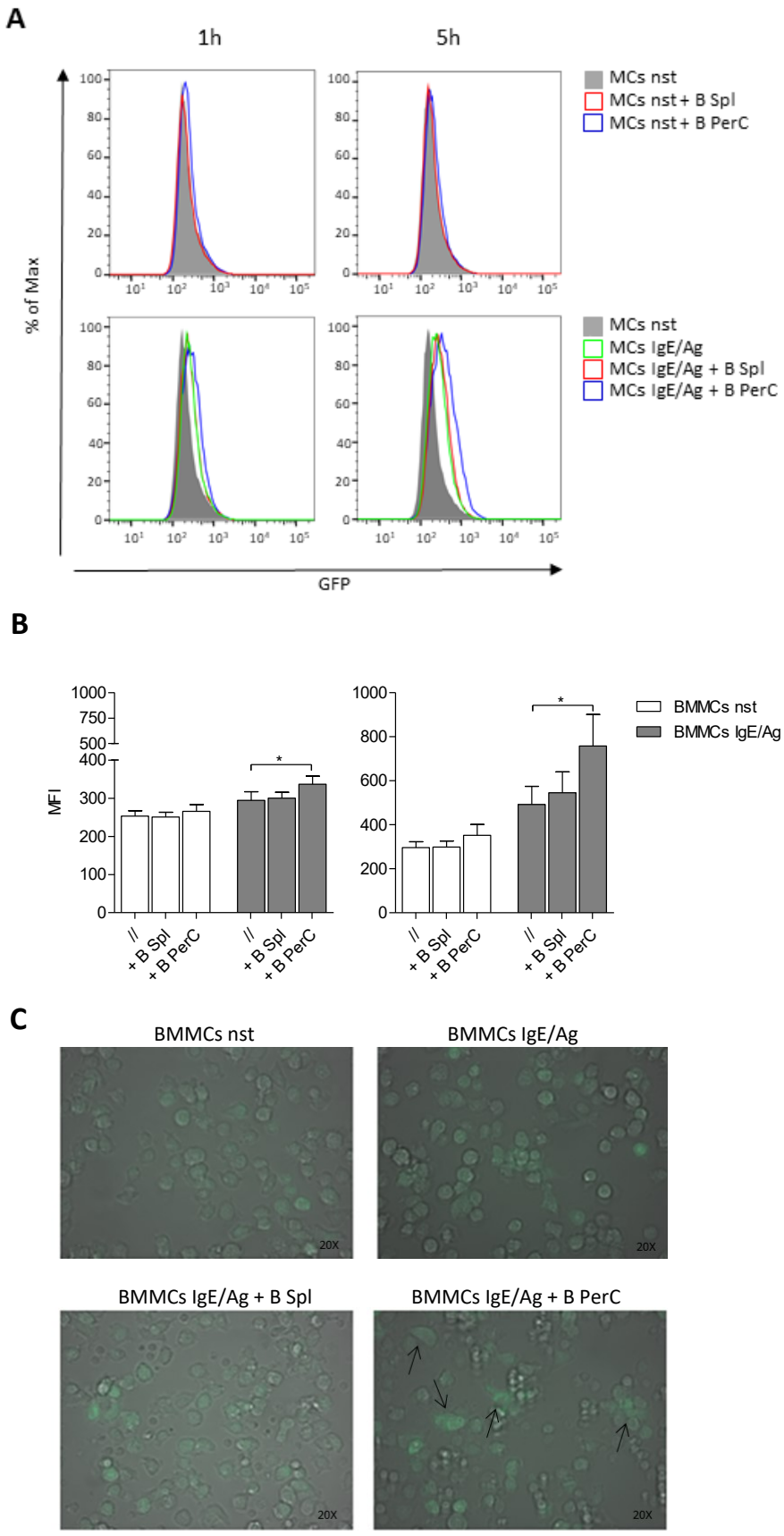


Figure 27: Peritoneal cavity B cells promote the increase of the green fluorescence in CCL2-eGFP BMMCs. CCL2-eGFP resting (nst) or IgE/Ag stimulated BMMCs, cultured for 1 or 5h with splenic (Spl) or peritoneal cavity (PerC) B cells in the presence of 2µM BFA, were analysed by flow cytometry and by fluorescent microscopy. **(A)** BMMCs, first selected on their morphology and for c-Kit positivity (not shown), were analysed for the expression of the GFP, indicative of the CCL2 protein accumulation in the cytoplasm. Representative histogram plots show all the conditions analysed. **(B)** Bar charts, mean of at least 3 independent experiments, show the mean fluorescence intensity (MFI) of the GFP in BMMCs in the different condition of culture. **p<0.01 by one-way ANOVA. **(C)** Representative microscopy images of the system taken at 5h. Magnificence=20x.

5.1.5. Soluble mediators arising from B-1 B cells are important for the potentiation of MCs' activation

Collectively, the obtained results indicate that peritoneal B cells play an important role in favouring and sustaining the allergic activation of MCs, potentiating their pro-inflammatory protein synthesis and release. We next decided to investigate whether this effect depends on a contact-mediated or soluble mediator-dependent mechanism. We chose to perform the following experiments by analysing the regulation of TNF-α production as representative since this MC-derived mediator is extremely important in both the early phases of inflammation and in tumor conditions, the two pathological process that are studied in the MC-B cell cross-talk *in vivo*.

We first hypothesized the existence of a membrane axis, established peculiarly with peritoneal and not with splenic B cells. The most known co-costimulatory pathway in the MC-B cell crosstalk is the CD40/CD40L axis[195,196]. However, the CD40 molecule is expressed at comparable levels between B-2 conventional lymphocytes and innate-like B-1 cells[295]. In literature, it is reported that a concurrent stimulation of MCs through CD28 and FcεRI induces an increase of TNF-α secretion in a dose-dependent manner[296]. Importantly, peritoneal B cells have a higher expression of CD80 and CD86 co-stimulatory molecules compared to splenic B cells[297]. Therefore, we hypothesized the instauration of a stronger CD28 - CD80/CD86 crosstalk between the MCs and peritoneal cavity B cells. To test this hypothesis, B/MCs co-cultures were set up and the signalling through CD28 was blocked using the non-stimulatory E18 Fab anti-CD28 Ab. However, as shown in *figure 28*, the secretion of TNF-α was not affected when this axis was blocked.

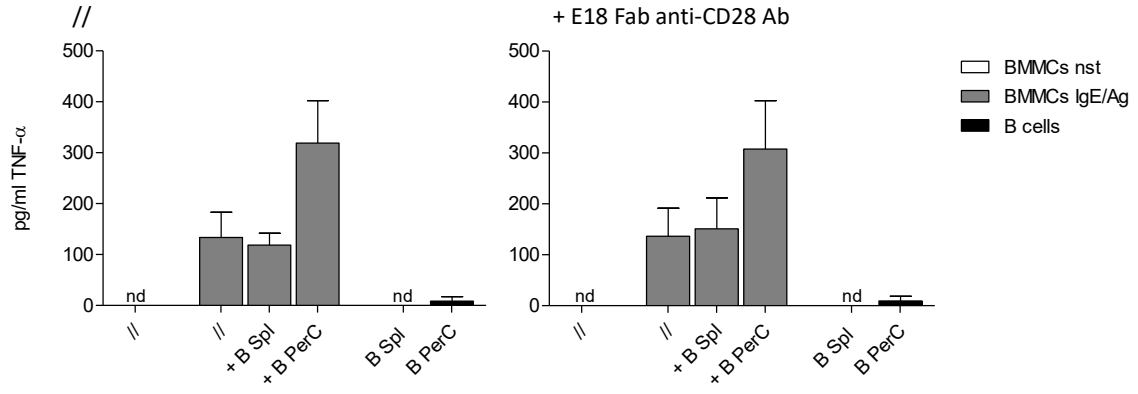


Figure 28: Analysis of TNF-α release by blocking the CD28 signalling in MCs in the MC-B cell co-culture. Secreted TNF-α from resting (nst) and activated (IgE/Ag) BMMCs alone or cultured for 6h with splenic (Spl) or peritoneal cavity (PerC) B cells is detected by ELISA. On the left normal medium was used in the co-cultures (//), on the right, the experiments were conducted by adding the E18 Fab anti-CD28 Ab. Reported results are mean (+SEM) of values from three independent experiments.

We next tested the hypothesis that soluble mediators released from freshly purified peritoneal B cells in their resting conditions could be responsible for the sustained neo-synthesis of TNF-α.

We explored this possibility by activating BMMCs in the presence of normal medium or of conditioned media derived from an 1h culture of either splenic or peritoneal B cells. Interestingly, the sole presence of the 1h conditioned medium derived from peritoneal cavity B cells given for 6h to IgE/Ag activated BMMCs was sufficient to sustain the previously described increase of TNF-α release (*figure 29A*). Differently from conventional B-2 B cells, B-1 B cells are able to produce a broad range of mediators without the need of stimulation. Indeed, our previously reported ELISA analysis showed that purified peritoneal B cells release IL-6, IL-10, low levels of TNF-α and CCL2 in steady-state (*figure 23*). Cell supernatants are rich in mediators of different nature and since MCs are sensitive not only to protein molecules but also to lipid mediators, the B cells-derived conditioned media derived from a 1h culture were boiled at 80°C in order to denaturate protein molecules. IgE/Ag activated BMMCs were cultured in the boiled media and the result of this assay was that the effect on TNF-α production was abrogated (*figure 29B)*. Therefore, this experiment suggest that mediators responsible for the effect of the B cells-derived conditioned media have a thermolabile nature, such as cytokines.

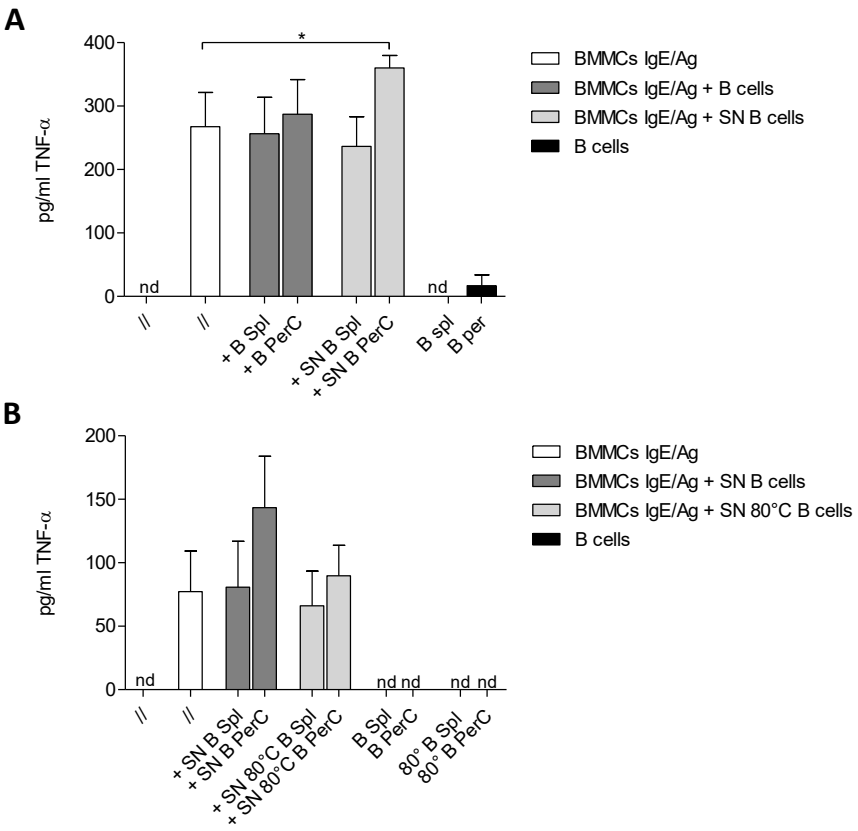


Figure 29: Soluble thermolabile mediators derived from peritoneal cavity B cells are able to promote TNF-α production in activated MCs. (A) Measurement of TNF-α release after 6h of culture of resting or IgE/Ag activated BMMCs cultured alone or in the presence of 1h conditioned supernatant (SN) from B cells. **(B)** IgE/Ag stimulated BMMCs were activated for 6h in the presence of 1h B cells' derived normal or of a SN boiled for 10 min at 80°C. Reported results are mean (+SEM) of values from three independent experiments. *p<0.05 by one-way ANOVA.

In the context of the analyses conducted to identify the molecular partners of the PerC B cell/MC interplay responsible for the switch towards an inflammatory phenotype of the MC, we hypothesized the involvement of the IL-33/ST2 axis. Specifically, we addressed the possibility that the mediators released from peritoneal cavity B cells induce IL-33 production in MCs which in turn is responsible for the increased membrane ST2 expression on MCs and synthesis of inflammatory cytokines. The choice to investigate the IL-33/ST2 axis was a consequence of our previous discovery that peritoneal cavity B cells induce an up-regulation of ST2 on MCs, and of literature data reporting that the IL-33 stimulation of MCs potentiates the production of pro-inflammatory mediators from IgE activated MCs[55,71]. According to our hypothesis, BMMCs cultured for 5h in peritoneal B cell-derived conditioned media present a slight increase of IL-33 gene transcription (*figure 30A*). However, this signalling pathway does not influence ST2 up-regulation nor TNF-α release, since IL-33 K.O. BMMCs in co-culture with peritoneal B cells act in the same way in terms of membrane ST2 induced up-regulation and TNF-α production and release (*figure 30B-C*).

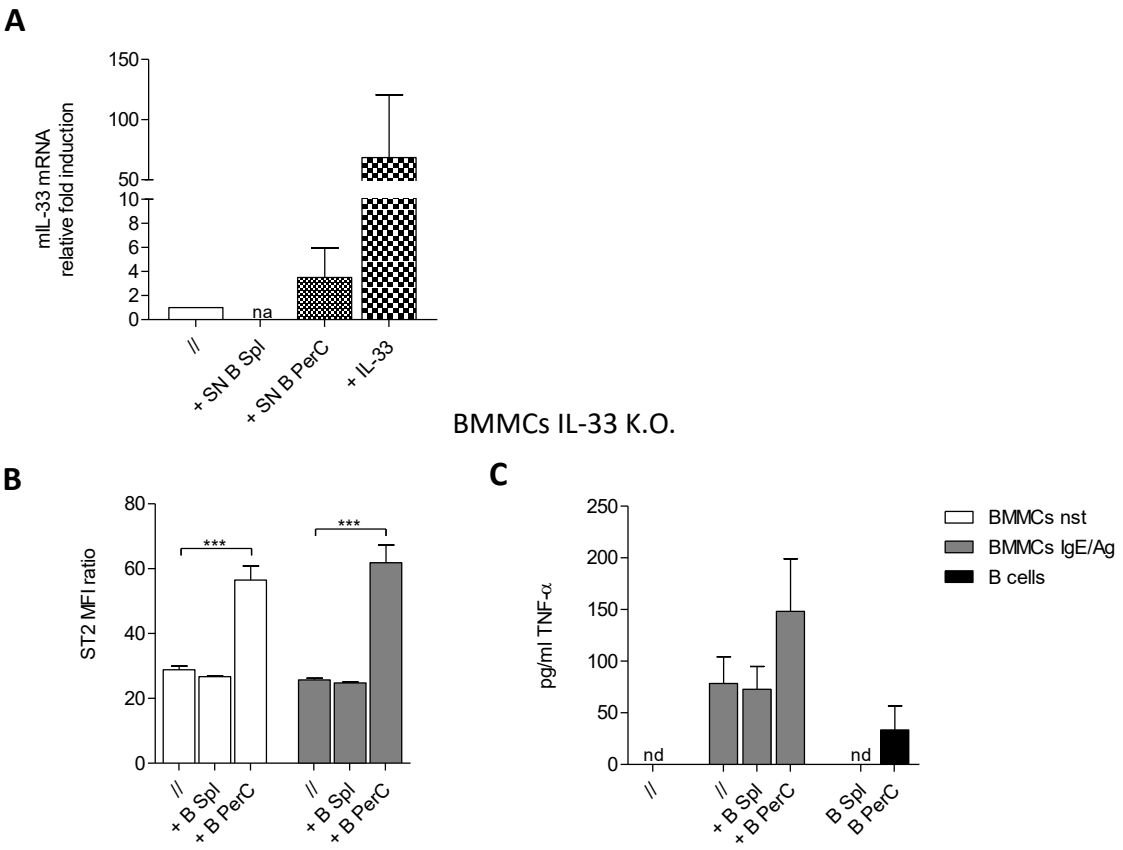


Figure 30: IL-33-dependent activation of MCs is not responsible for the increase of membrane ST2 and TNF-α neo-synthesis. (A) Wt BMMCs were cultured for 5h in normal medium or in the 1h supernatant (SN) derived from either splenic (Spl) or peritoneal cavity (PerC) B cells. The graph reports il33 mRNA gene expression expressed as fold induction over not stimulated (//) MCs. MCs stimulation with recombinant IL-33 was used as a positive control[63]. **(B)** c-Kit$^+$ resting (nst) or IgE/Ag

activated BMMCs from IL-33 K.O. mice were analysed by flow cytometry for the expression of membrane ST2 after 24h of co-culture with splenic (+B Spl) or peritoneal cavity (+B PerC) B cells. **(C)** TNF-α was detected by ELISA after 24h of IL-33 K.O. MCs culture in the absence or presence of B cell subpopulations (splenic=Spl; peritoneal cavity=PerC). One sample Student's t-test (A) and one-way ANOVA (B,C) have been used. na= not amplified, nd=not detected.

Altogether, this first part of our data demonstrates an intriguing and specific cross-talk established between MCs and peritoneal cavity B cells. We can argue that peritoneal cavity B cells are essential during an acute or chronic allergic activation of MCs since they exacerbate the release of pro-inflammatory mediators by MCs. A wider screen is needed to determine the specific factor (or the combination of more mediators) responsible for the observed effect. For example, IL-10, that is highly released by peritoneal B cells, has been shown to sustain and potentiate the allergic activation of MCs[298].

We can conclude that, *in vivo*, the peritoneal cavity and the intestine, which are known to be colonized by both MCs and innate-like B lymphocytes[299], should be considered as important anatomical sites where an exacerbation of MCs detrimental activation may be controlled by acting on B-1 lymphocytes.

5.1.6. MCs' distribution in *bumble* B1-lacking mice

Our *in vitro* study concerning the interplay between MCs and B-1 versus B-2 B cells highlighted an important role for peritoneal B cells in sustaining MC's activation. For this reason, we were interested in determining if the presence or the absence of B-1 cells could affect MCs distribution and phenotype *in vivo*. To our knowledge, two types of B-1 cell-deficient animal models are available: μ−/− mice reconstituted with the conventional B-2 cell population[200] or the *bumble* model, a mutated mouse in which the impaired expression of the atypical IκB protein IκBNS drastically reduces the frequencies of B-1 cells in these animals[130]. Thanks to the collaboration with the group of Prof. Karlsson Hedestam GB., we analysed the role of B-1 B cells on the distribution and phenotype of MCs in *bumble* mice. As a first step we decided to assess the percentages and absolute numbers of peritoneal MCs and, as a control, of the B cell subsets. The combination of anti-CD19, anti-CD23 and anti-CD5 mAbs was used to follow the peritoneal B cell subsets (see methods *paragraph 7.3.3*) while anti-c-Kit plus anti-FcεRI mAbs to identify peritoneal MCs. Through this phenotypical analysis, we confirmed that the mice were B-1-lacking (*figure 31A*), and we found that the relative numbers of peritoneal MCs are not affected in the mutated *bumble* mouse (*figure 31B*).

Moreover, the expression of FcERI and c-Kit phenotypical markers on MCs are not significantly altered in the absence of B-1 cells (*figure 31C*). We next examined other MCs-populated tissues: the intestine (duodenum, jejunum and ileum) and the lungs. In collaboration with the Tumor Immunology Unit led by Prof. Tripodo C., we performed two types of histological stains on the paraffin sections of the tissues of interests: Hematoxylin and Eosin (H&E), to obtain information about the architecture of the tissues, and Toluidine Blue, to detect MCs which are red-purple cells in a blue background. Compared to the normal tissue situation of the Wt samples, all the *bumble* mouse tissues analysed had signs of inflammation, hyperplasia and increased immune infiltrates, as illustrated in *figure 32*. Concerning MCs distribution, a significant increase of MCs was observed in the lungs and an increase in the jejunum portion of the intestine (*figure 33*). The pleural cavity and the intestine are known to be a B-1 B cells colonized sites and the increase of MCs in these anatomical compartments suggests that the absence of B-1 cells somehow affects the normal distribution of MCs. Together, all these analyses indicate that these mice had some abnormalities of the immune cells with an increase of local spots of inflammations directly or indirectly due to the severe reduction of the B-1 cell subsets and that B-1 cells may have a role in regulating MCs distribution in the tissue analysed. To determine any role of B-1 cells on MCs phenotype and activation further analyses are needed.

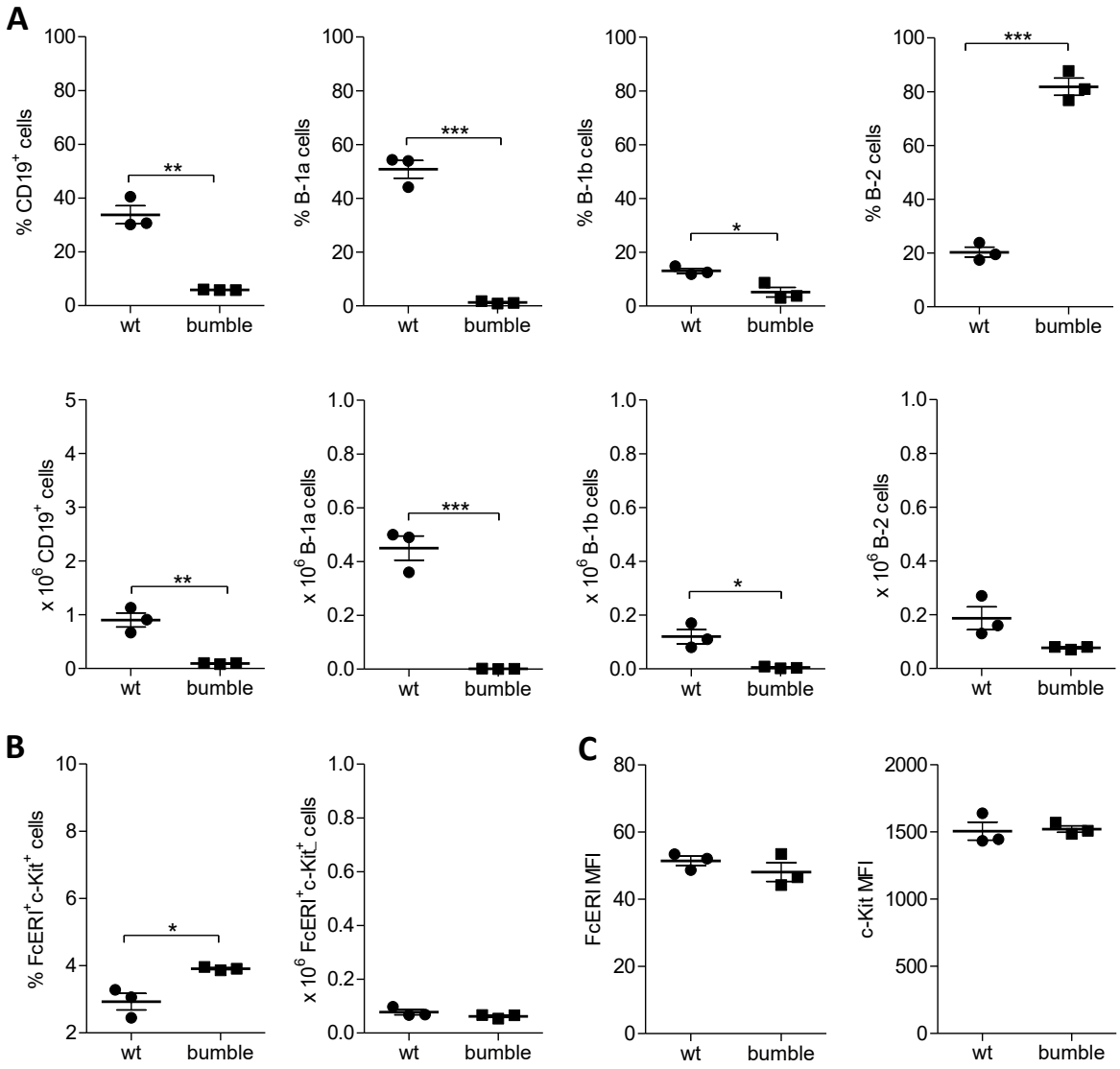


Figure 31: B cells and MCs counts in peritoneal lavages from bumble mice. (A) Peritoneal lavages from Wt and *bumble* mice were stained with anti-CD19, -CD23 anti -CD5 mAbs and analysed by flow cytometry. B cells subsets were identified as: total B cells ($CD19^+$ cells), B-1a ($CD19^+CD5^+CD23^-$), B-1b ($CD19^+CD5^-CD23^-$) and B-2 ($CD19^+CD5^-CD23^+$). Percentages among total peritoneal cells are indicated in the upper panels, absolute numbers are shown in the lower panels. **(B)** $Fc\varepsilon RI^+c\text{-}Kit^+$ peritoneal MCs are shown as % and absolute numbers among total cells. **(C)** The MFI for FcεRI and c-Kit receptors on MCs are shown for both the two groups of animals. Each symbol depicts individual mice among the Wt and *bumble* groups (n=3). $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ by two-tailed Student's t-test.

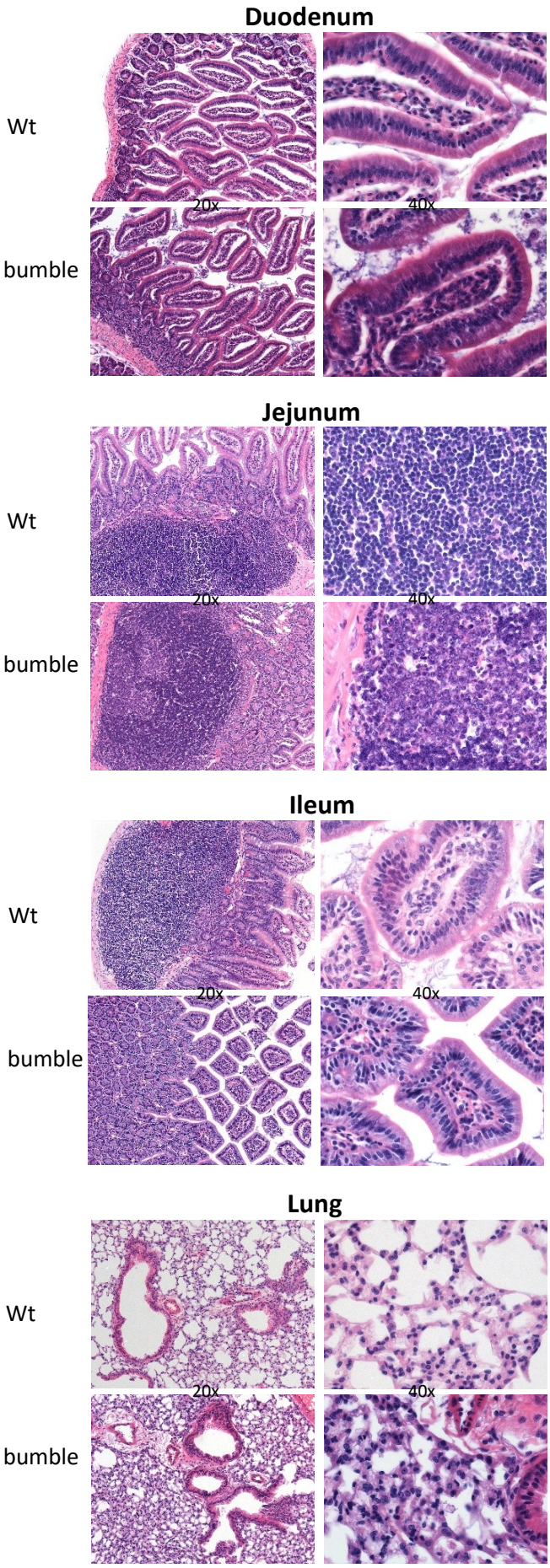


Figure 32: Representative pictures of H/E-stained tissues of Wt and *bumble* mice. Tissue samples were recollected from 3 *bumble* and 3 control mice (Wt). Small intestine (composed of duodenum, jejunum and ileum) and lung sections were stained with Hematoxylin/Eosin (H&E). Images were taken with 20x (pictures on the left) and 40x magnificence (pictures on the right) with an optical microscope.

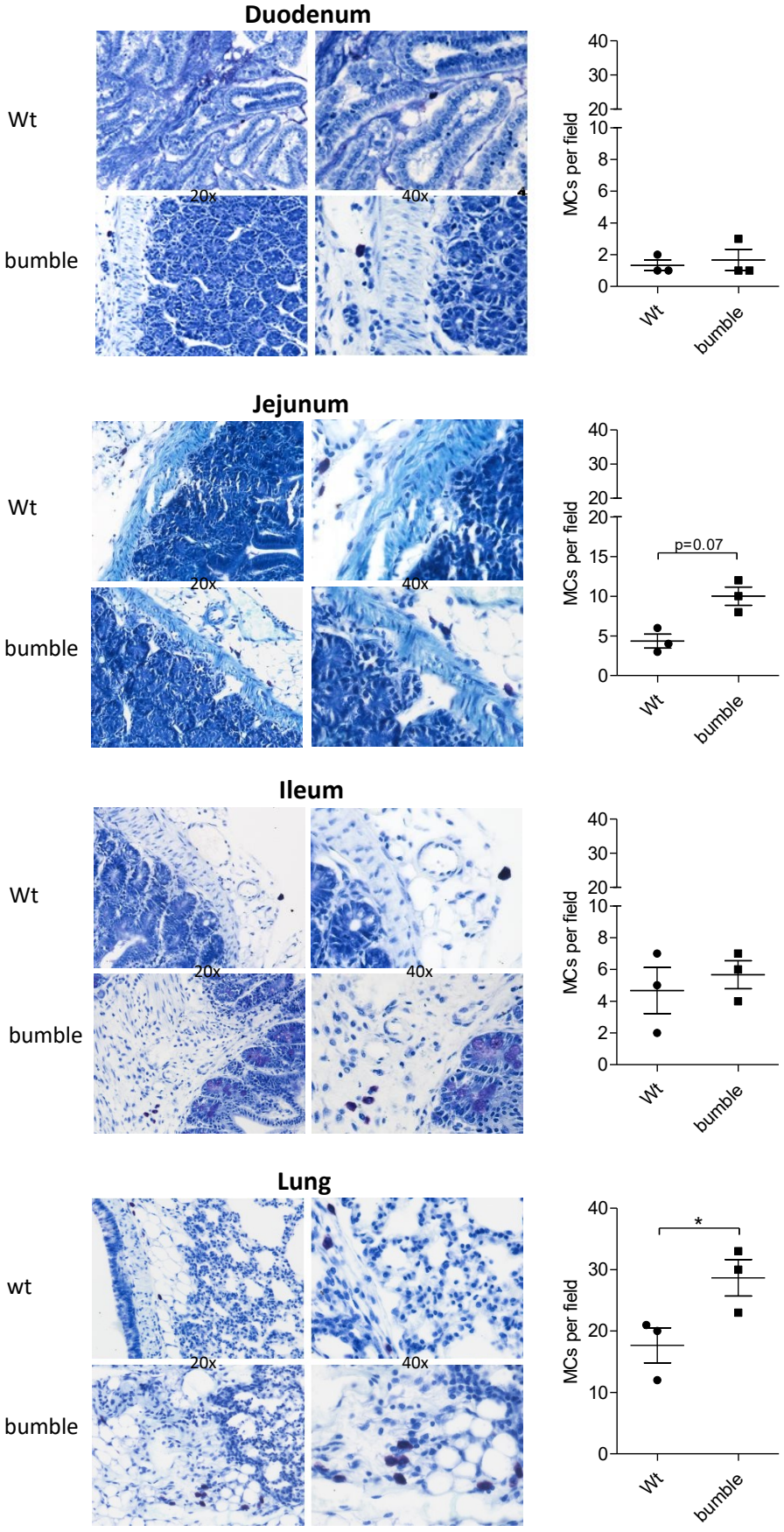

Duodenum
Wt
bumble
20x
40x
MCs per field
Wt
bumble
Jejunum
Wt
bumble
20x
40x
p=0.07
MCs per field
Wt
bumble
Ileum
Wt
bumble
20x
40x
MCs per field
Wt
bumble
Lung
wt
bumble
20x
40x
*
MCs per field
Wt
bumble

Figure 33: MCs organ distribution in Wt and B-1 cell-lacking *bumble* mice. Tissue samples were recollected from 3 *bumble* and 3 control mice (Wt) and Toluidin Blue staining for MCs was performed. Representative histological images are shown. The graphs indicate the number of MCs per field analysed in all mice samples. $^{*}p<0.05$ by two-tailed Student's t-test.

5.2. MC-B CELLS BIDIRECTIONAL CROSS-TALK UNDER PHYSIOLOGICAL CONDITIONS: THE B CELL SIDE

5.2.1. B-2 B cells activation and antibody production are sustained by MCs

T helper lymphocytes are the most important B cells' partners since they are able to give proliferative priming and to induce specific antibody responses[300]. However, in a T-independent B cell activation, infective soluble mediators or pro-inflammatory cytokines released by other immune cell types are able to promote B cell survival and activate them into effector cells. Previous works by our group showed that BMMCs induce both survival and proliferation of naive splenic B cells and that they can lead to the switch of B cells into effector IgA-producing cells. Both cell-to-cell contacts (where the CD40/CD40L axis covers a relevant contribute) and soluble mediators released by stimulated BMMCs (among which IL-6 was shown to be highly important) have been discovered to co-operate for the sustaining of the survival and proliferation of B cells and to their switch into IgA-producing cells[195].

Because of the importance of considering the different local microenvironments in the study of the cross-talk between two different immune cell types, we decided to extend our previous study and compare the effect of MCs on the survival and proliferation not only of B-2 B cells but also of B-1 B cells. Therefore, freshly purified splenic or peritoneal cavity B cells were cultured alone or with either unstimulated or IgE-Ag-activated MCs, in a 1:1 ratio. Panel A of *figure 34* shows the results of our analysis of B cell viability. The amount of viable splenic B cells is lower if compared with peritoneal B cells (respectively about 20 and 50%). In accordance with the previously published data[195], the culture in the presence of both resting and activated MCs supports B-2 B cells survival. Conversely, the viability of peritoneal B cells is not affected by the presence of MCs. Similarly, the proliferation of splenic B cells is favoured by the presence of MCs while peritoneal cavity B cells are not induced to proliferate by effect of MCs (*figure 34B*). The explanation for our evidences that peritoneal B cells do not require the support of MCs for their survival and proliferation could be inherent in the fact that B-1 cells are evolutionary self-sustaining and self-renewing.

Next, we decided to explore the possibility that MCs could modulate the expression of B cell receptors important for their status of activation. Among the different pathways, we decided to focus on CD80 and CD86 co-stimulatory receptor molecules that are expressed on APCs and are up-regulated by inflammatory and antigen-specific signals (Greenwald, Freeman, & Sharpe, 2005). Moreover, the CD80/86-CD28 is a membrane co-stimulatory axis that can be established between the two cell types. Ag-derived peptides are bound and presented through the MHC-II complex expressed constitutively in APCs such as B cells. Programmed cell death 1 (PD-1), as well as its ligand PD-L1, are shown to be expressed on B cells. This axis affects cell proliferation, growth and apoptosis and is studied for its modulation of cell tolerance in terms of inhibition of immune responses in cancer. Moreover it has been shown that the transient expression of PD-1 on Ag-experienced B-1b cells suppresses their *in vitro* proliferation and IgG polarization, in the absence of other co-stimulations[301].

Under resting conditions, CD80, and to a lesser extent CD86, are more expressed by peritoneal cavity B cells compared to splenic B cells. PD-1 is also expressed with more intensity by peritoneal cavity B cells while MHC-II has comparable levels of expression on the two B cell subtypes. While CD80 expression on both B cell types is not affected after co-culture with MCs, CD86 and MHC-II, as previously shown by Palm and co-workers[198], are modulated on the B-2 population by the presence of MCs. Conversely, peritoneal cavity B cells do not change their basal expression of the markers after co-culture with MCs. Interestingly PD-1 is downregulated on splenic B cells in the presence of MCs (*figure 34C*). Regarding this last result, we can speculate that, similarly to what happens with B-1b cells[301], the decreased expression of PD-1 following the interaction with the MC could promote cell proliferation. On the contrary, higher levels of PD-1 on peritoneal B cells may explain their "unavailability" to proliferate in the presence of MCs' mediators and cell contact.

Igs, which are exclusively produced by B cells, are essential to protect vertebrates from infections and are the preponderant component of the immunological memory. The formation of long-lived memory B cells and PCs that produce high-affinity, isotype-switched Abs crucial for host defence is the emblem of humoral immunity[160]. In light of the evidences that MCs promote B cells' humoral responses in the context of the B-2 B cell population[195,198], we decided to carry on our study by comparing the amount of IgM, IgG, IgA, IgE released in the media after 48h in both the B Spl/MC and B PerC/MC co-culture systems. Panel D of *figure 34* shows the result of our analysis for the sole IgM, IgG and IgA isotypes since no IgE release was detectable in our system. The presence of IgE/Ag triggered MCs favours an augmented release of all the three Ab isotypes analysed compared to

basal levels on splenic B cells. On the contrary, once again, no MC's contribution has been appreciated for peritoneal cavity B cells' Ab production.

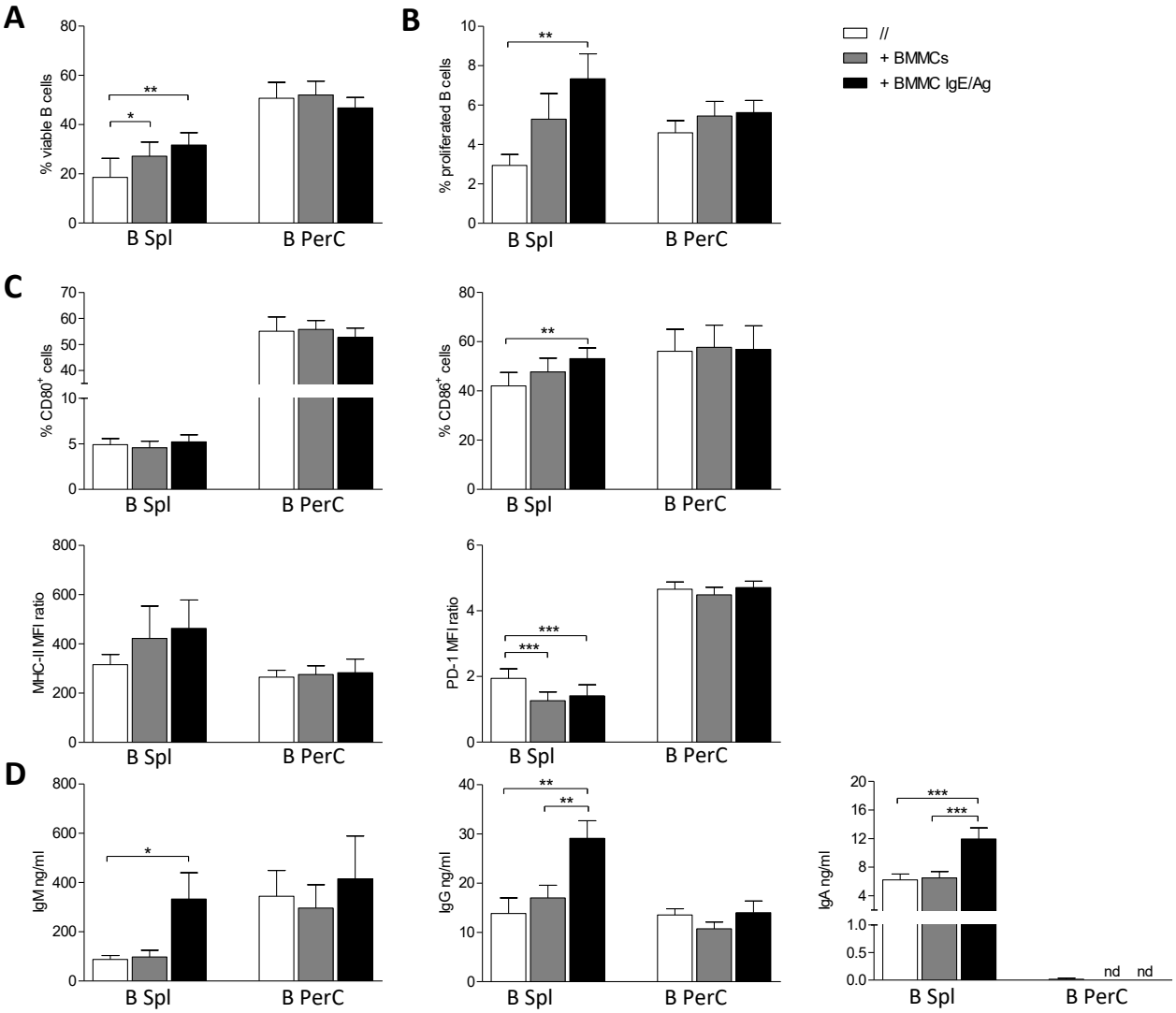


Figure 34: MCs sustain the activation of the B2-eriched B cell population and have no impact on peritoneal B cells' functions. (A) Naive splenic (Spl) or peritoneal cavity (PerC) B cells, respectively enriched in B-2 and B-1 cells, were cultured for 48h in the presence of resting (+ BMMCs) or activated (+ BMMCs IgE/Ag) MCs. Percentages of viable B cells were determined by flow cytometry following Annexin V/Propidium Iodide staining. **(B)** CFSE-labeled splenic or peritoneal B cells were cultured for 72h either alone or in the presence of non-sensitized or activated BMMCs. The percentages of B cells with diminished CFSE intensity were detected among total $CD19^+$ cells by flow cytometry. **(C)** Naive splenic and peritoneal B cells, cultured for 48h in the presence of non-sensitized or activated BMMCs, were stained for PD-1, CD80, CD86 and MHC-II complex and analysed by flow cytometry. Percentages of $CD19^+$ cells expressing the markers are reported in the analysis for CD80 and CD86, MFI ratio is indicated in the MHC-II and PD-1 analysis. **(D)** Splenic and peritoneal cavity B cells were cultured for 48h in the presence of resting or stimulated (IgE/Ag) MCs. Soluble Igs were quantified by ELISA. Means (+SEM) from at least three independent experiments are shown. $^*p<0.05$, ** $p<0.01$, *** $p<0.001$ by one-way ANOVA. nd = not detectable.

5.2.2. B cell-related chemokine receptors are modulated by MCs

In pathological conditions activated B cells can leave the lymphoid organs and accumulate in inflammatory sites[302]. The work of Palm, suggested that MCs may enhance the capacity of B cells to home lymphoid tissues and inflammatory sites by modulating their L-selectin membrane expression[198]. In this light, we decided to determine if MCs are able to modulate B-cell related chemokine receptors involved in chemotaxis. After 48h of MC-B cell co-culture CCR4, CCR6, CCR9 and CCR10 chemokine receptors were analysed on $CD19^+$ cells by flow cytometry. We focused on CCR6, CCR9 and CCR10 since it is known that these receptors drive the migration of B cells and of activated PCs in intestinal lymphoid structures both in physiology and in inflammation. The CCR4 skin-related receptor was analysed as a control (*figure 35*). Among the receptors analysed, the basal expression between cultured B-2 and B-1 cells is comparable. Our results indicate that, when activated, MCs may influence the chemotactic properties of B cells by modulating their chemokine receptors. Specifically, while CCR10 is not affected by MCs in both B cell subsets, CCR9 and CCR4 expression are decreased on splenic B cells. This result can be either a decreased expression because of the binding of the receptor to its chemokine ligand (CCL25 for CCR9 and CCL17 for CCR4) hypothesizing that MCs has released these chemokines, or the polarization of B-2 cells' migration in different anatomical sites. Conversely, activated MCs induce the up-regulation of CCR6 (the receptor of CCL20) on both B cells subtypes. As mentioned in the introduction of this thesis, CCL20, drives the migration of B cells in both homeostatic and inflammatory conditions, mostly in intestinal lymphoid structures. MCs can therefore play a role in facilitating B cells migration towards a CCL20 gradient.

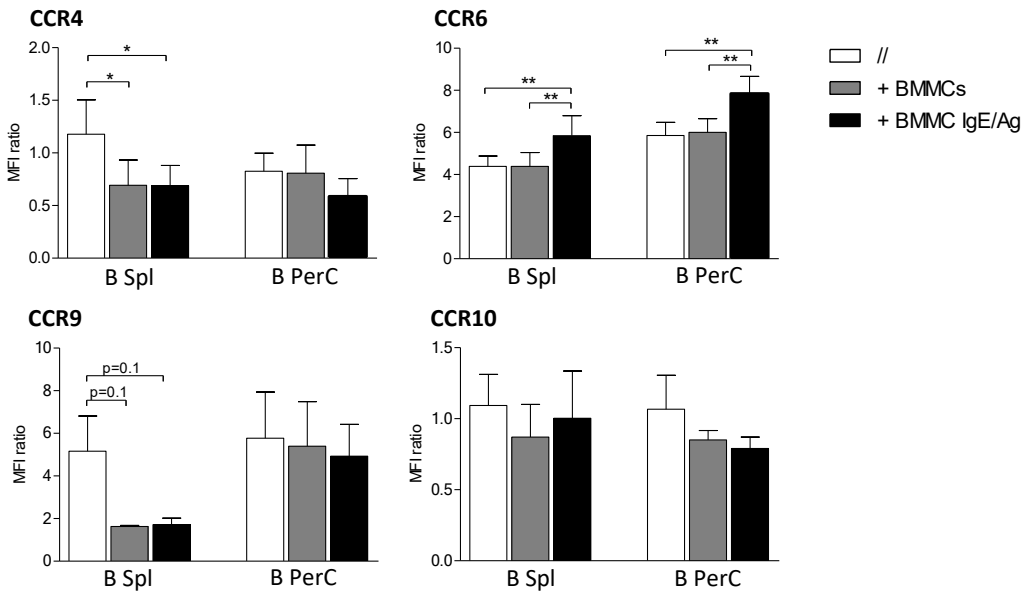


Figure 35: Activated MCs are able to induce the up-regulation of CCR6 chemokine receptor on both splenic and peritoneal cavity B cells. Naive splenic (Spl) and peritoneal cavity (PerC) B cells were cultured alone (//) or in the presence of resting (+ BMMCs) or activated (+ BMMCs IgE/Ag) MCs for 48h. $CD19^+$ cells were analysed for the expression of CCR4, CCR6, CCR9 and CCR10 chemokine receptors by flow cytometry. MFI ratio is indicated in the analysis. Means (+SEM) from at least three independent experiments are shown. $^*p<0.05$, $^{**}p<0.01$ by one-way ANOVA.

5.3. THE B CELL/MC CROSS-TALK UNDER PHYSIOLOGICAL CONDITIONS: B CELLS DISTRIBUTION, PHENOTYPICAL CHARACTERIZATION AND ACTIVATION IN MC-DEFICIENT MICE MODELS

Although the role played by MCs in supporting B cell related functions is widely accepted, to our knowledge the impact of the absence of MCs on B cell subsets distribution, phenotype and effector properties under physiological conditions has not been investigated in detail. To explore this condition, we decided to characterize B cells in different lymphoid organs by comparing two MC-deficient mice models: the *Kit*$^{W\text{-}sh}$ mouse, that, as extensively described in the introduction, lacks mature MCs in all body tissues because of the mutation a the level of the Kit gene, and the RMB (Red Mast Cell and Basophil) mouse in which the tracking of both MCs and basophils is possible by the red fluorescence of the td-Tomato (tdT) protein. In our experiments we compared on the one hand Wt C57BL/6 mice, *Kit*$^{W\text{-}sh}$ mice and *Kit*$^{W\text{-}sh}$ mice i.p. reconstituted with BMMCs, and, on the other, RMB mice with DT-treated-RMB mice (DT-RMB). From all mice we sampled the blood, spleen, peritoneal cavity lavage, lymph nodes (LNs) and colon and performed B cell-related analysis. In order to ascertain that MCs' depletion or reconstitution occurred, the presence of FcεRI^{+}c-Kit^{+} cells was checked by flow cytometry.

5.3.1. MCs do not affect the phenotypical distribution of B cells in different lymphoid organs

We first determined the distribution of B cell subsets in the spleen, peritoneum and in a pool of mesenteric, inguinal and axillary LNs. On the basis of the differential expression of the CD21 and CD23 surface markers, splenic B lymphocytes can be divided into three main populations: newly formed (NF) CD21^{-}CD23^{-}, marginal zone (MZ) CD21^{high}CD23^{-} and follicular (FO) CD21^{+}CD23^{+} B cells. Moreover, the staining with anti-CD19, -CD21 and -CD23 mAbs allows also the identification of CD21^{low} transitional 2-marginal zone precursors (T2-MZP) B cells. The CD1d^{hi}, CD1d^{+}CD5^{-}, CD1d^{+}CD5^{low} and CD1d^{+}CD5^{+} subsets were determined through the staining with anti-CD19, -CD1d and -CD5 Abs (methods *section 7.3.2*). Anti-CD19, -CD23, -CD5 Abs were instead used to follow the B-1a, B-1b and B-2 peritoneal cavity B cell subsets (as described before). For all these stainings, a representative gating strategy is reported in the methods *section 7.3.2* and *7.3.3.* Interestingly, both the MC-deficient mouse models analysed showed that total B cell percentages were not altered in the absence of MCs in none of the anatomical sites investigated in our experiments. Of note, total

peritoneal cavity B cells were slightly increased upon reconstitution of *Kit$^{W\text{-}sh}$* mice (*figure 36A*). Similarly, the B cell distribution in the different splenic phenotypical subsets was not affected by the absence of MCs (*figure 36B*). A discrepancy between the two MC-deficient mouse models emerged instead from the analysis of the B cell distribution in the peritoneum. While in the RMB system no differences were observed in the peritoneal B cell subsets between RMB and DT-RMB mice, an inverted proportion of B-1 and B-2 B cells was uncovered in *Kit$^{W\text{-}sh}$* mice which presented a considerable increase (from 40% to 80%) of conventional B-2 B lymphocytes (*figure 36C*). However, upon MCs reconstitution of *Kit$^{W\text{-}sh}$* mice the B-1 and B-2 distribution is unchanged, indicating that the presence of MCs within the peritoneal cavity is not sufficient to restore the Wt condition.

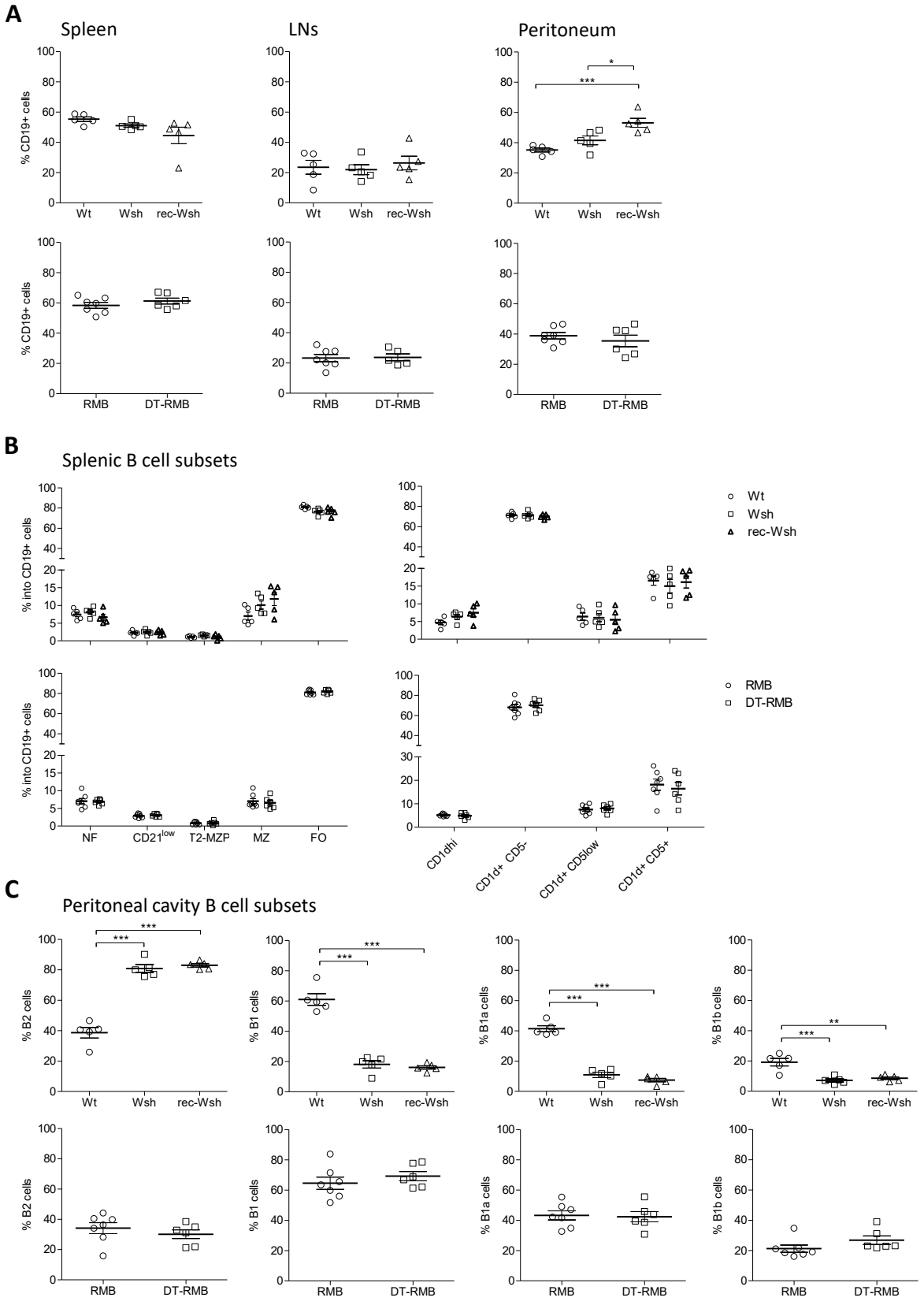


Figure 36: B cell subsets distribution in lymphoid organs in MC-deficient mice models. Upper graphs of each figure section A, B and C compare Wt, Wsh and BMMCs-reconstituted rec-Wsh mice. Lower panels compare instead RMB with MCs-depleted RMB (DT-RMB) mice. Cells were isolated from the different organs of the mice genotypes and analysed by flow cytometry. **(A)** The

frequencies of B cells in the spleen, lymph nodes (LNs) and peritoneum are shown as $CD19^+$ cells. **(B)** Splenocytes were isolated and stained with anti-CD21, -CD23 and -CD19 (histograms on the left) or with anti-CD1d, -CD5, and -CD19 (histograms on the right) mAbs and analysed by flow cytometry. $CD19^+$ cells with the phenotype of newly formed (NF), $CD21^{low}$ transitional 2-marginal zone precursors (T2-MZP), marginal zone (MZ) and follicular (FO) cells or of $CD1d^{hi}$, $CD1d^+CD5^-$, $CD1d^+CD5^{low}$ and $CD1d^+CD5^+$ are shown in the graph as percentages among the total B cell population. Each symbol indicates individual mice among the groups analysed. **(C)** Peritoneal lavages from the Wt and MCs-deficient mice were stained with anti-CD19, -CD23 and -CD5 mAbs and B cell subsets were identified as B-2, B-1a and B-1b cells.
Each symbol depicts individual mice among the different groups $^*p<0.05$, $^{**}p<0.01$, *** $p<0.001$ by two-tailed Student's t-test (for RMB mice) or one-way ANOVA (Wsh mice).

5.3.2. Splenic B cells' antibody production is affected in the absence of MCs

Since we observed that B cell frequencies in MC-lacking mice are not altered by the absence of MCs, we next investigated if markers related to B cells' activation were affected. In the light of the fact that our *in vitro* data showed that MCs provide a support for only the B-2 enriched splenic B cell population, we decided to analyse the expression of membrane CD80, CD86, MHC-II and CD138 only on B cells obtained from the spleen. As shown in *figure 37A*, no differences were observed in the expression of the activation markers analysed in both the MC-deficient mouse models under investigation, suggesting that in physiological conditions MCs are not responsible for the modulation of these molecules on B cells.

Considering these results, we decided to investigate whether the absence of MCs *in vivo* "primed" B cells to respond differently to a T cell-dependent stimulation (mimicked by an anti-CD40 mAb) or to a bacterial bioproduct (LPS) or to a viral stimulation (CpG). To this aim, purified B cells were kept in culture in normal medium or with the aforementioned stimuli and cell proliferation and viability were analysed as readouts. As shown in *figure 37B*, *ex vivo,* B cells from normal and MC-deficient mice responded in the same way to the stimulations in terms of cell survival and proliferation.

Since one of the major effects of MCs on B cell biology is related to Ab production, we next assessed whether the absence of MCs *in vivo* "primed" B cells to respond differently to anti-CD40 mAb, LPS or CpG in terms of IgM, IgG and IgA production. Cell supernatants were collected after 48h of culture and Ab concentrations were detected by ELISA. Interestingly, in the $Kit^{W\text{-}sh}$ system, the absence of MCs seems to slightly affect the B cell capacity to secrete the IgM and IgG isotypes, especially in resting conditions and in response to the a-CD40 mAb for IgM and in response to LPS stimulation in the case of IgG. This effect was due to the absence of MCs and not the Kit mutation *per se* since B cells purified from the spleens of reconstituted $Kit^{W\text{-}sh}$ mice recovered the ability to produce IgM

and IgG at their normal extent. A similar tendency was observed for the analysis of the IgA released by stimulated B cells. Indeed, *in vivo*, the presence of MCs is required for the full capability of B cells to respond to stimulation and produce IgA. Moving to the RMB mouse model, a different scenario was uncovered. No differences between normal and MC-deficient mice were observed for the IgM and IgG isotypes. On the contrary, B cells purified from MC-depleted DT-RMB mice released higher levels of IgA in both the presence and absence of stimulation.

These results altogether suggest that MCs have a role in the regulation of Ab production in homeostatic conditions. The differences observed between the two models can lie in the fact that MCs are absent from the birth of the animal in the $Kit^{W\text{-}sh}$ model while in the RMB model MCs are depleted in the adult life.

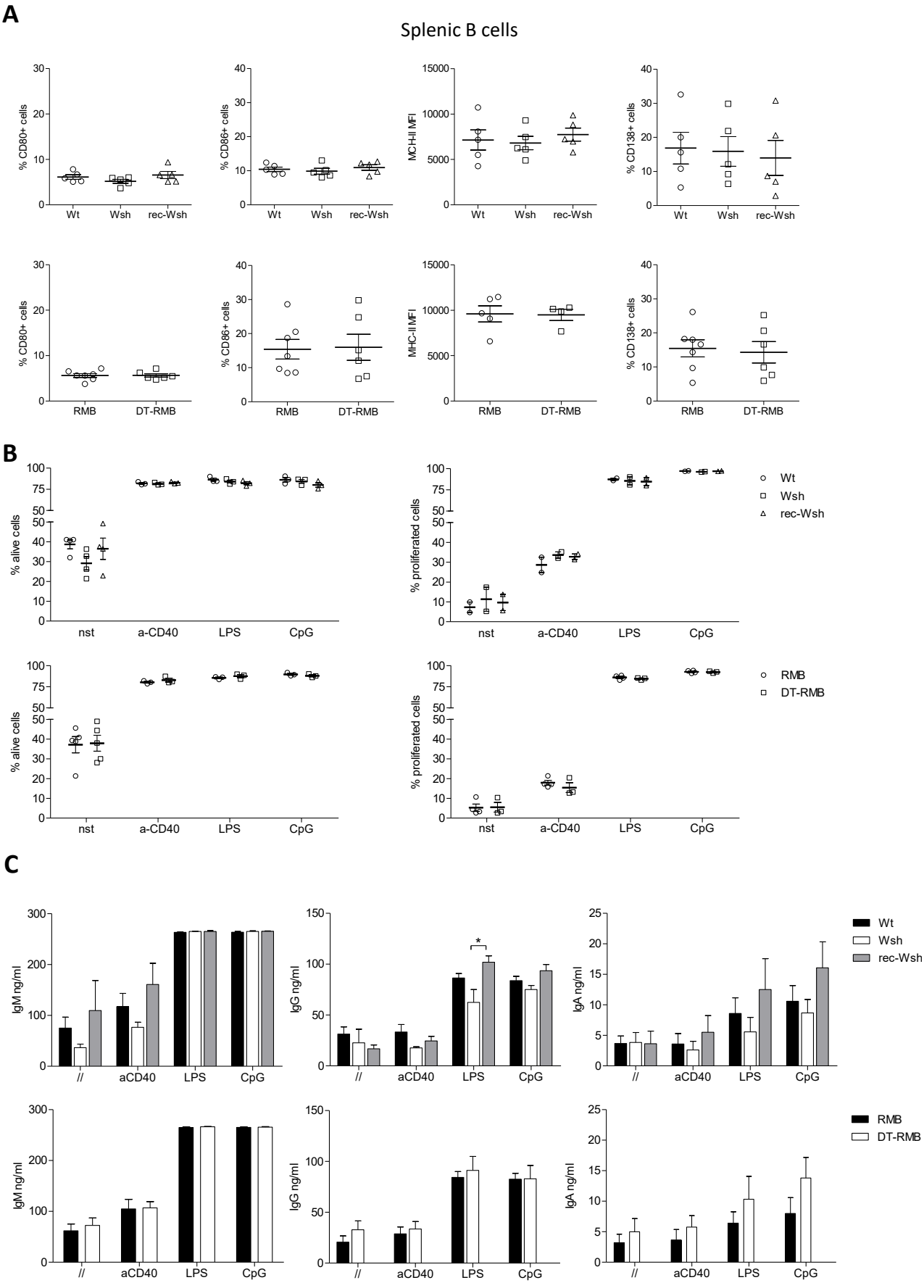


Figure 37: Analysis of splenic B cells' phenotype, survival, proliferation and Ab production from MC-deficient mice in homeostatic conditions. (A) Isolated splenocytes from Wt, Wsh and rec-Wsh mice (upper

graphs of each panel) and from RMB and DT-RMB mice (lower graphs) were analysed by flow cytometry. Among the $CD19^+$ population, the % of CD80 and CD86 positive cells was identified based on control isotypes. MHC-II expression on $CD19^+$ cells is indicated as MFI. Splenic B cells were purified through a beads-based negative selection and $CD138^+$ cells were identified as % based on control isotypes in the $CD45R^+$ and $CD45^{int}$ stained population. **(B)** B cells were cultured either alone (nst) or in the presence of a-CD40 mAb, LPS or CpG for 48h. Percentages of viable B cells were determined by flow cytometry following Annexin V/Propidium Iodide staining (graphs on the left). CellTrace Violet Cell Proliferation Dye-labeled splenic B cells were cultured for 72h either alone or in the presence of a-CD40 mAb, LPS or CpG. The percentages of B cells with diminished dye intensity, indicative of proliferation, were detected among total $CD19^+$ cells by flow cytometry. **(A, B)** Each symbol depicts individual mice among the different groups. **(C)** Naive splenic B cells were cultured for 48h in the presence or absence of a-CD40 mAb, LPS or CpG and afterwards supernatants were collected for the following IgM, IgG and IgA sandwich-based ELISA measurements. Means (+SEM) from at least three independent experiments are shown. $^*p<0.05$ by two-tailed Student's t-test (for RMB mice) or one-way ANOVA (W-sh mice).

In order to explore the results we obtained in the analysis of Abs produced by splenic B cells *ex vivo*, we detected IgM, IgG and IgA Ab isotypes also at the systemic level, in the blood.

The analysis of seric Abs was in line with the results relative to *ex vivo* splenic B cells' antibody production. No significant difference was observed in IgG concentration in the blood of $Kit^{W\text{-}sh}$ and DT-RMB mice in respect to the normal counterpart. IgM was tendentially decreased in the $Kit^{W\text{-}sh}$ model and recovered in rec-$Kit^{W\text{-}sh}$ mice compared to the Wt condition. Instead, in the RMB system, we detected an opposite tendency, with an increase of IgM titres in the absence of MCs. Finally, IgA concentrations were higher in both $Kit^{W\text{-}sh}$ and DT-RMB mice with respect to the MC-suffcient conditions, although the reconstitution of $Kit^{W\text{-}sh}$ mice did not restore Wt condition (*figure 38A and B*). Concerning the RMB model, to ascertain that IgA and IgM modifications were MC-dependent, and not a consequence of the injection of the DT itself in mice, we injected i.p. either PBS or DT into C57BL/6 recipient mice (B6) and serum was analysed through ELISA. No increase of IgA or IgM was observed in DT-treated B6 mice supporting the evidence of a role for MCs in the regulation of Ab responses in healthy mice (*figure 38C*).

Altogether these results indicate that, although MCs do not affect B cells distribution in different lymphoid organs nor their expression of activatory molecules, they cover a significant role in the modulation of Abs production by B cells.

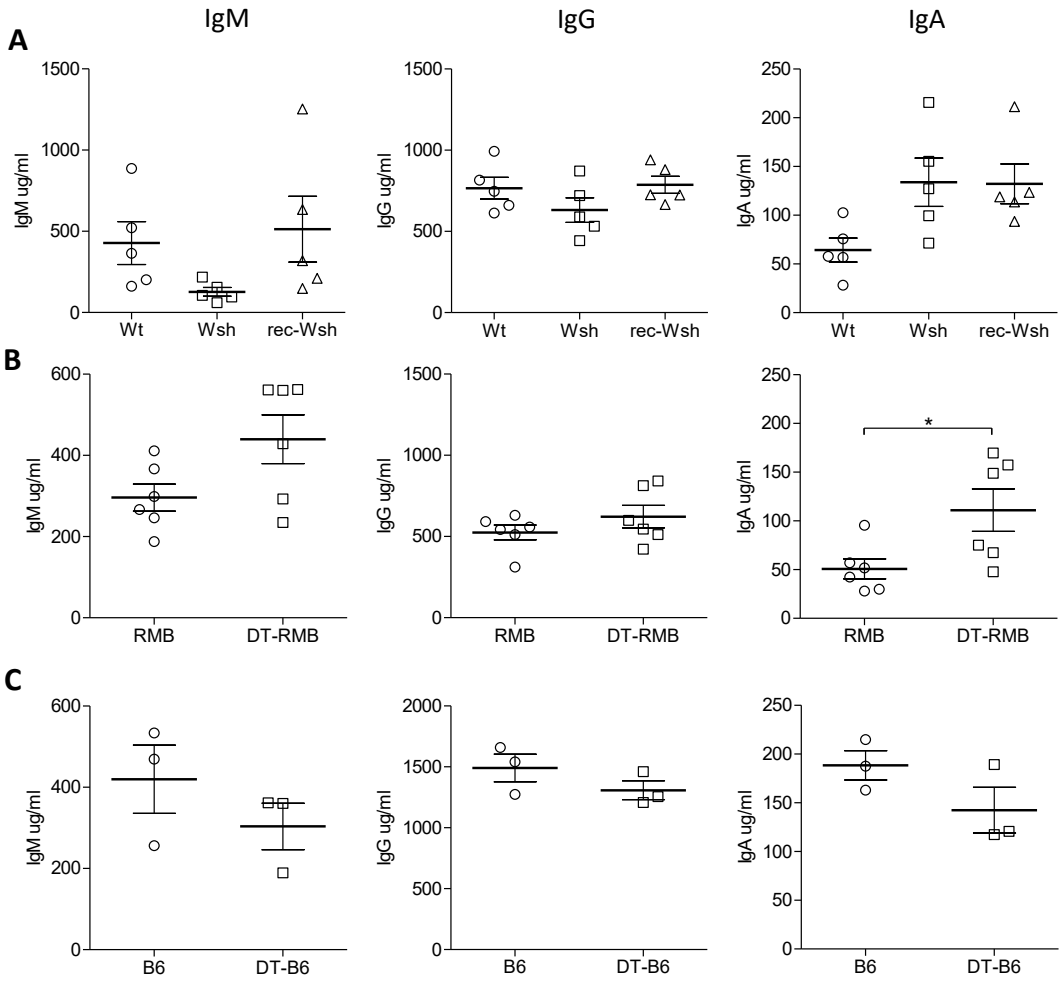


Figure 38: Seric antibodies in Wt and MC-deficient mice in homeostatic conditions. IgM, IgG and IgA concentrations were measured in blood samples of Wt, Wsh and rec-Wsh (**A**), RMB and DT-RMB (**B**) mice and in Wt C57BL/6 animals injected either with PBS (BS) or DT (DT-B6) **(C)**. *p<0.05 by two-tailed Student's t-test (for RMB mice) or one-way ANOVA (W-sh mice).

5.4. THE B CELL/MC CROSS-TALK UNDER PATHOLOGICAL CONDITIONS: B CELLS DISTRIBUTION, PHENOTYPICAL CHARACTERIZATION AND ACTIVATION IN THE DSS-RMB MOUSE MODEL

After getting an overview of B cells distribution and functional properties in MCs-depleted mice under homeostatic conditions, we further explored the effect of this immune cell type on B cell behaviour in a pathological condition. Specifically, we analysed the B/MC cross-talk in the context of intestinal inflammation since the intestinal compartment is an important anatomical site in which MCs and B cells were shown to co-localize in physiology and in IBD patients[195,196]. In this view, we set up the model of the dextran sodium sulfate (DSS): 2% DSS was added to the normal drinking water and this induced acute colitis in our mice. Among the two available MC-deficient mouse models we decided to explore the RMB model since the analyses conducted in physiological conditions in the *Kit$^{W\text{-}sh}$* model highlighted additional anomalies in the B cell compartment (such as in the peritoneal cavity) that are independent from the presence of MCs since upon reconstitution the Wt condition is not restored. In addition, the DSS-induced inflammation has never been studied in the RMB mouse context. Since our aim was to investigate if during inflammatory conditions B cell subpopulations and functionality were affected, we decided to reproduce the most relevant analyses conducted in healthy conditions.

5.4.1. DSS-induced intestinal inflammation in the RMB mouse model: overview

Before analysing B cell behaviour we decided to evaluate the disease progression in the RMB model since, compared to Wt mice, a worse prognosis of intestinal inflammation was reported for *Kit$^{W\text{-}sh}$* animals following DSS administration[233]. In light of literature data reporting a reduction of colon length following the establishment of colitis[303], the analysis of this parameter was chosen as an indicator for evaluating the status of inflammation. F*igure 39A* shows that, as expected, DSS administration induced a reduction in colon length in both RMB and DT-RMB mice compared to their control counterparts. However, colon length was inferior in DSS-treated DT-RMB with respect to RMB mice, confirming the data emerging from the *Kit$^{W\text{-}sh}$* model. In support to the colon length result, the analysis of weight loss in DSS-treated RMB and DT-RMB mice showed that MC-depleted mice had a worse progress of inflammation since, at the day of the sacrifice, they had lost more weight compared to the RMB counterpart (*figure 39B*). In addition, a more intense rectal bleeding in the DT-RMB mice was noticed (not shown). Finally, we also observed that mesenteric LNs (MLNs)

size was increased in both RMB and DT-RMB DSS-treated mice compared to healthy controls because of the direct effect of inflammation (not shown).

To sum up, in accordance with previously reported data[233], in the DSS-RMB mouse model the absence of MCs has a negative impact on the disease. Therefore, this aspect must be kept in mind when analysing the results regarding the impact of the absence of MCs on B cell behaviour during intestinal inflammation.

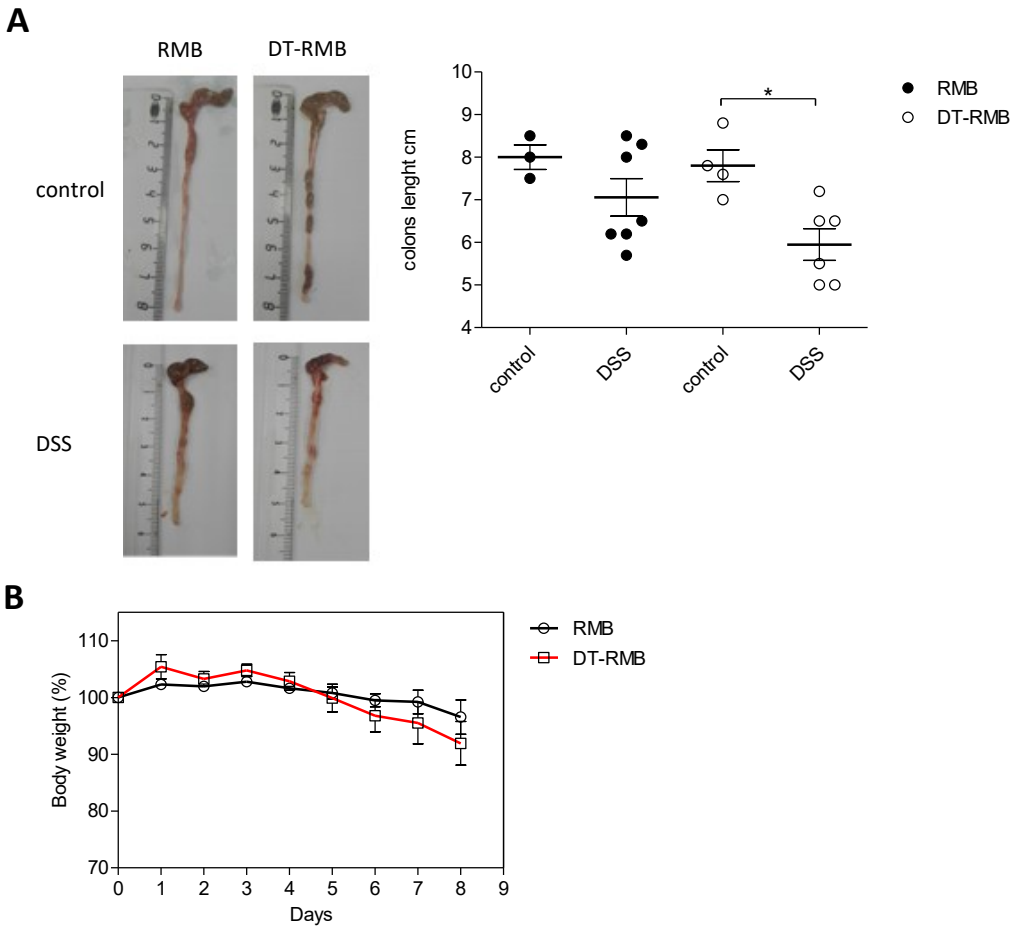


Figure 39: DSS-induced colitis has a worse progression in DT-RMB mice. (A) Colon lengths were measured and are indicated in cm. **(B)** Weight analysis of RMB and DT-RMB 2% DSS-treated mice is expressed as % of the starting weight of individual mice. Each symbol indicates individual mice. $^{*}p<0.05$ by one-way ANOVA.

5.4.2. B cell distribution and phenotype during intestinal inflammation is not altered in the absence of MCs

Concerning B cell distribution in spleen, MLNs and peritoneum, no differences in B cells percentages were observed between DSS-treated RMB and DT-RMB mice (*figure 40A*). While the distribution of

splenic B cell subsets was not altered with the progression of colitis, peritoneal B-1 cells were slightly increased in the B-1b component in the absence of MCs condition (*figure 40B-C*). We next analysed CD80, CD86, MHC-II and CD138 membrane markers on splenic B cells to determine whether, differently from the homeostatic condition, the absence of MCs in inflammation could influence the activation status of B cells. However, in the two mice analysed, no differences were observed (*figure 40D*). Finally, splenic purified B cells were further investigated in their *ex vivo* survival rate but also this feature was not altered in DT-RMB mice (*figure 40E*).

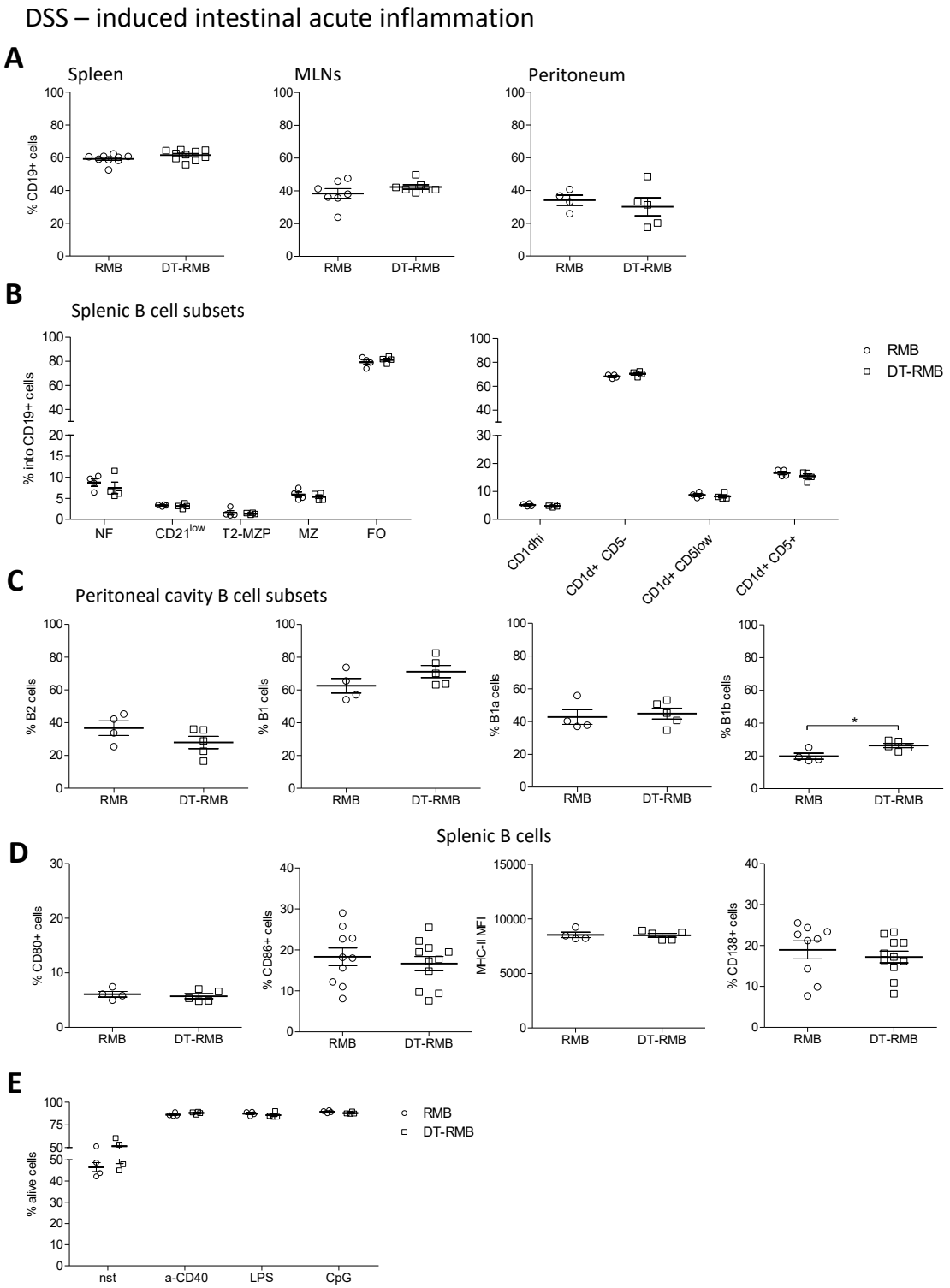


Figure 40: Panoramic of B cells' distribution, phenotype and survival in DSS-induced colitis in the RMB model. (A) The frequencies of B cells are shown for the spleen, mesenteric lymph nodes (MLNs) and peritoneum as $CD19^+$ cells. **(B)** Splenocytes were stained with anti-CD21, -CD23 and -CD19 (left panel) or with anti-CD1d, -CD5, and -CD19 (right panel) mAbs and analysed by flow cytometry. $CD19^+$ cells with the phenotype of newly formed (NF), $CD21^{low}$, transitional 2-marginal zone precursors (T2-MZP), marginal zone (MZ) and follicular (FO) cells (graphs on the left) or of $CD1d^{hi}$, $CD1d^+CD5^-$, $CD1d^+CD5^{low}$ and $CD1d^+CD5^+$ (graph on the right) are shown in the graph as percentages among the total B cell population. **(C)** Peritoneal lavages from DSS RMB and DT-RMB mice were stained with anti-CD19, -CD23 and -CD5 mAbs and B cell subsets were identified as: B-2, B-1a and B-1b cells. **(D)** Isolated splenocytes from DSS RMB and DT-RMB mice were analysed by flow cytometry. Among the $CD19^+$ population, the

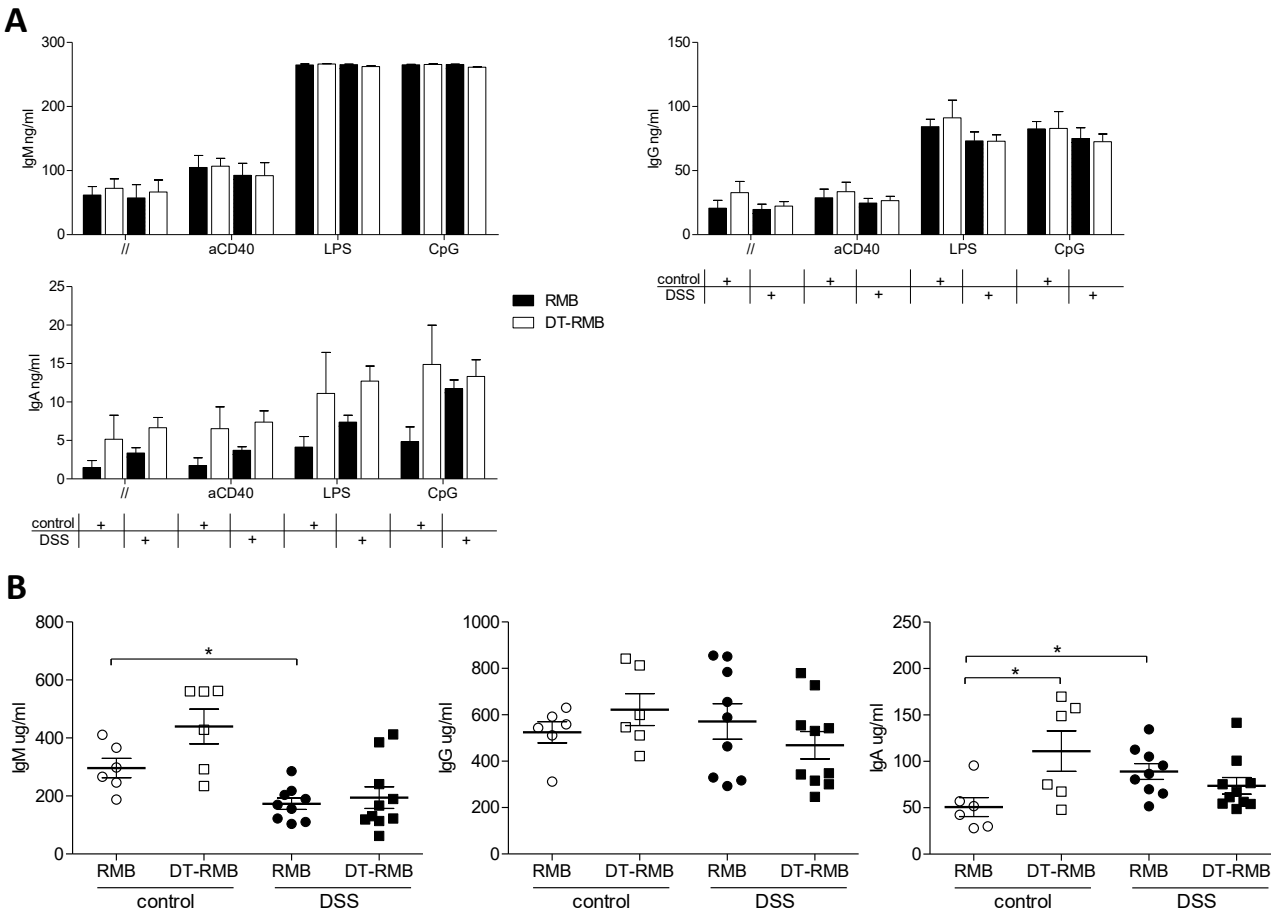
% of CD80 and CD86 positive cells was identified based on control isotypes. MHC-II expression on $CD19^+$ cells is indicated as MFI. $CD138^+$ cells are identified as % based on control isotypes in the $CD45R^+$ and $CD45^{int}$ B cell population. **(E)** Purified splenic B cells were cultured either alone (nst) or in the presence of a-CD40 mAb, LPS or CpG for 48 h. Percentages of viable B cells were determined by flow cytometry following Annexin V/Propidium Iodide staining. Each symbol depicts individual mice among the different groups (panels A, B, C, D and E), $^*p<0.05$ by two-tailed Student's t-test. Means (+SEM) from at least three independent experiments are shown in panel F.

5.4.3. IgA increase in DSS mice is positively regulated by the presence of MCs

We next examined *ex vivo* Ab production from splenic purified B cells. Among the Ab isotypes, IgA analysis is particularly interesting in the context of the intestinal inflammation since it has been reported that higher level of protective IgA are produced to face intestinal inflammation and to protect mucosal surfaces[242,243]. Our analysis of Ab production, in the presence or absence of stimulation, showed that IgM and IgG are not affected DT-RMB mice compared to the RMB controls. Concerning the IgA isotype, what we observed upon DSS treatment was that while splenic B cells from RMB mice increased their IgA production both in resting condition and upon stimulation, B cells from DT-RMB mice did not increase upon DSS treatment (*figure 41A).*

Clearer and robust data concerning the contribution given by MCs in sustaining B cells IgA production during colitis are also shown in *figure 41*. Indeed, following DSS administration, IgA levels were significantly increased in RMB mice but not in DT-RMB mice if compared to their respective healthy controls. This effect seems to be peculiar of the IgA isotype since serum levels of IgG were unchanged in DSS RMB and DT-RMB mice compared to their respective physiological controls, while on the contrary serum IgM levels are significantly diminished (*figure 41B*). In order to further investigate this result, we assessed the accumulation of IgA^+ PCs at the site of colonic inflammation by performing immunohistochemistry analyses. Interestingly, in homeostatic conditions no differences in the numbers of IgA^+ elements between RMB and DT-RMB colons were observed. However, following DSS treatment, RMB mice presented an increase in the number of PCs, which we did not observe in DT-RMB mice (*figure 41C*), suggesting again that the lack of MCs might affect the correct increase of IgA^+ PCs that are important actors in the resolution of the inflammation. Altogether these results led us to hypothesize that, during the establishment of the colitis, B cells of MC-depleted mice lack the factor given by MCs that support their IgA class switching. It is interesting to note that our data indicate that MCs play a role in the sustaining of the IgA switching only in inflammatory conditions, since in homeostasis, on the contrary, an increase in the IgA production

has been observed in the absence of MCs. We can speculate that resting MCs in homeostatic conditions provide factors that control the production of IgA by B cells while, when they are activated in the context of an acute inflammation, such as colitis, the contact of the two cells and the released mediators favour the expansion of IgA^+ PCs and their Ab release.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IgM ng/ml
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aCD40
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C
IgA
control
DSS
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40X
Counts of IgA+cells
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Figure 41: IgA production in DSS treated mice is supported by the presence of MCs. (A) Naive splenic purified B cells were cultured for 48h in the presence or absence of a-CD40 mAb, LPS or CpG and supernatants were collected for ELISA measurements. In the graphs, both the homeostatic and the DSS analyses are plotted together. **(B)** IgM, IgG and IgA concentrations were measured in serum samples of DSS RMB and DT-RMB by ELISA. Results are plotted in comparison with the analysis of healthy controls of respective mice. Each symbol indicates individual mice among the groups analysed. **(C)** Representative immunohistochemistry analysis of anti-IgA immunostaining of formalin-fixed paraffin-embedded colon sections from control and DSS RMB and DT-RMB mice. Counts of IgA^+ elements are also provided. Each symbol of the scatter plot is the mean count of 5 fields (40x). $^*p<0.05$ by two-tailed Student's t-test or one-way ANOVA. ns=not significant.

5.5. THE B CELL/MC CROSS-TALK UNDER PATHOLOGICAL CONDITIONS: B CELL BIOLOGY IN A SUBCUTANEOUS MODEL OF CRC

Focusing our attention on another pathological context we tried to decipher the contribution of MCs for B cells' activation in the context of CRC. The MC is a relevant cell type to consider when analysing tumor development and several therapeutic approaches targeting MCs have been attempted for different cancer types[304]. The choice of analysing this type of cancer is due to the fact that the accumulation and activation of MCs in CRC have been thoroughly investigated and it has emerged that MCs accumulation at the tumor edge have a prognostic significance[248].

Recently, the characterization of the modifications in the distribution, phenotype and effector functions of B lymphocytes during CRC progression has been object of interest of the research group in which this work of thesis has been carried on. Specifically, an extensive analysis has been conducted by comparing three models of CRC: the genetic $Apc^{Min/+}$ mouse, the colitis-associated CRC induced by AOM/DSS, and BALB/c mice subcutaneously injected with the CT-26 cell line[263] .

In order to investigate the role of the MC in the changes observed in the B cell population during CRC onset and progression, we first injected the MC-38 colon adenocarcinoma cell line in the right flank of C57BL/6 mice (for further details about the procedure see *methods 7.2.1*) and performed a characterization of B cells' distribution, phenotype and Ab production comparing tumor-bearing mice with the PBS-injected counterpart. The properties of B cells in Wt tumor-bearing mice were then compared with those observed in $Kit^{W\text{-}sh}$ and in i.p. BMMCs-reconstituted $Kit^{W\text{-}sh}$ -tumor bearing mice. The MC is defined as an antenna of the microenvironment and, in this light, the interactions established between MCs and colon cancer cells might induce changes in the phenotype and functionality of MCs that could be relevant for the different behaviour of the B cell population in the tumor context. For this reason, the last part of these results section concerns the *in vitro* characterization of the cross-talk between MCs and the MC-38 colon adenocarcinoma cell line.

5.5.1. The frequencies of $CD19^+$ cells are increased in tumor draining LNs

First, we aimed at understanding if the growth of the MC-38 tumor could promote any variations in B cell numbers in different lymphoid organs and in the tumor site itself by effect off the micro- and macroenvironments generated upon cancer development. Interestingly, while no differences

(compared to controls) were observed in the B cell numbers in the spleen and peritoneum, a significant increase of $CD19^+$ elements was found in tumor-draining LNs (dLNs). Moreover, B cells were detectable also within the tumor, although at lower numbers compared to $CD4^+$ cells (*figure 42A*). In order to investigate whether, at a peripheral level, B cells acquire a peculiar tumor-dependent phenotype, we analysed the expression of surface markers related to B cell activation (CD80, CD86, MHC-II) and tumor-related molecules (PD-1, PD-L1, FasL) on splenic B cells. Interestingly, among the markers analysed, splenic B cells isolated from tumor-bearing mice differentially expressed MHC-II and PDL-1 (*figure 42B*). Next, we assessed whether splenic B cells have an impaired capacity to produce Igs, and therefore purified splenic B cells were cultured for 48h in presence or absence of a-CD40 mAb, LPS or CpG. We observed a trend toward a higher basal release of all the three Ab isotypes (IgM, IgG and IgA) from tumor-bearing mice purified splenic B cells. Moreover, although not significant, an increased release of IgA was observed also upon CD40 mAb, LPS and CpG stimulations (and of IgM in the presence of CD40 mAb) (*figure 42C*).

Altogether, these data indicate that, B cells are shaped and activated in the context of tumorigenesis, not only at the tumor site but also in different lymphoid organs. Moreover, the higher percentages of $CD19^+$ cells detected in dLNs suggest that B cells are induced either to proliferate or to be recruited in anatomical sites directly affected by the establishment of the tumor.

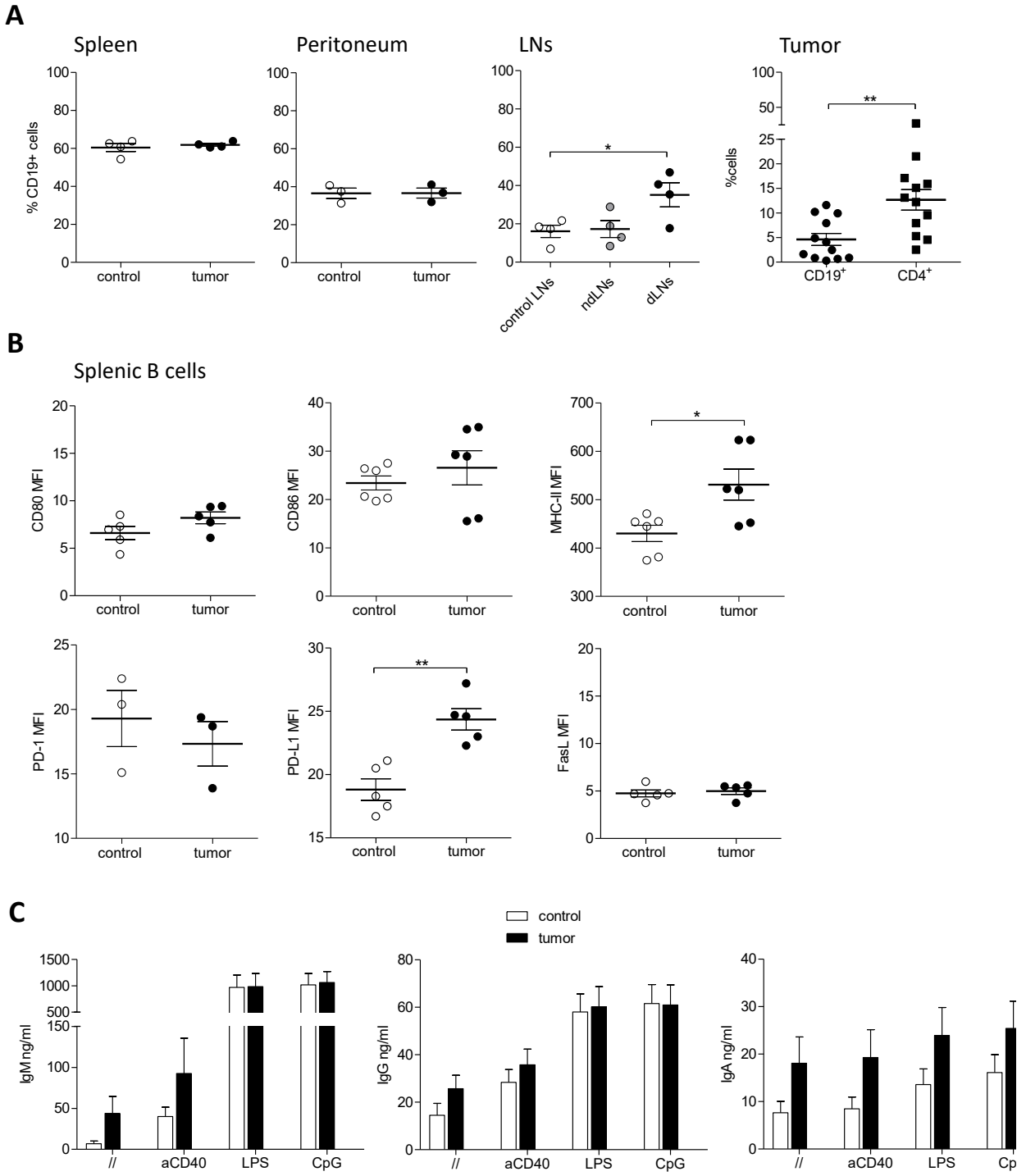


Figure 42: B lymphocytes are expanded in tumor-draining LNs of MC-38 tumor mice and infiltrate the tumor. (A) Percentages of $CD19^+$ cells were analyzed by flow cytometry in cell suspensions obtained from the spleen and peritoneum of control and tumor-bearing mice and in control or draining (d) and non-draining (nd) LN. B lymphocytes were also identified by flow cytometry after tumor digestion and compared with tumor-infiltrating $CD4^+$ T cells (far right panel). **(B)** $CD19^+$ splenocytes from control and tumor-bearing mice were analysed for membrane expression of CD80, CD86, MHC-II, PD-1, PD-L1 and FasL and results are indicated as MFI. **(C)** IgM, IgG and IgA were detected by ELISA in supernatants of 48h cultured resting or stimulated (anti-CD40 mAb, LPS, CpG) purified splenic B cells from at least 3 control and 3 tumor-bearing mice.
Each symbol depicts individual mice among the control and tumor-bearing groups. Analyses were done by means of two-tailed Student's t-test or by one-way ANOVA, *p<0.05, **p<0.01.

5.5.2. Higher chemotactic densities are observed in tumor dLNs

We already proposed that the enlargement of the B cell population in tumor dLNs is a generalized mechanism related to tumor onset and we excluded an increased proliferative rate[263]. In the present thesis, we addressed two different hypothesis considering chemotactic-related mechanisms: increased levels of LNs-related-B cell-chemokine receptors and an alteration of B cell-related chemokines in tumor dLNs.

To investigate the first hypothesis we analysed the expression levels of CXCR4, CXCR5, CCR7, CCR6 on splenic B cells by flow cytometry and the results we obtained was that no relevant differences in chemokine receptors expression between healthy and tumor-bearing mice is present (*figure 43A*). We then moved to the second possibility and analyzed mRNAs expression of the cxcl12, cxcl13, ccl21, ccl19, and ccl20 chemokine in LNs of tumor-bearing and control mice. Very interestingly, a significant increase of CXCL13 and CCL20 expression was observed in dLNs with respect to the control mice LNs (*figure 43B*), suggesting that these two chemokines could be responsible for the accumulation of B cells observed in tumor dLNs.

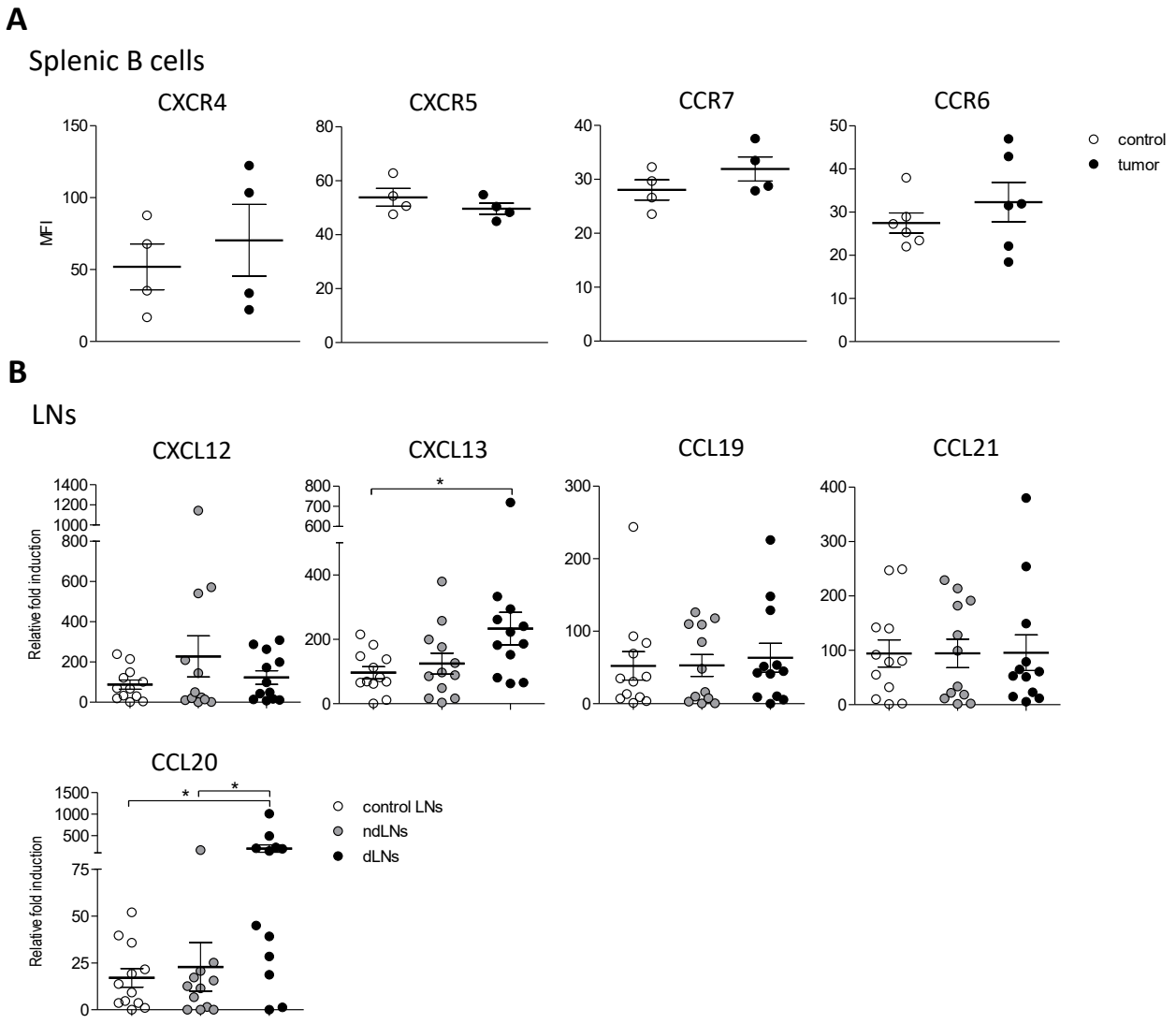


Figure 43: Increased expression of CXCL13 and CCL20 in dLNs of MC-38-tumor mice. (A) Surface expression of CXCR4, CXCR5, CCR7 and CCR6 chemokine receptors was analysed by flow cytometry on $CD19^+$ splenocytes isolated from control and MC-38 tumor-bearing mice and the. MFI are indicated in the graphs. **(B)** The relative expression of cxcl12, cxcl13, ccl19, ccl21 and ccl20, normalized to the housekeeping gene g3pdh, was analysed by qPCR in the total cellular population of LNs isolated from control and tumor-bearing mice. LNs from tumor mice were distinguished between non-draining (ndLNs) and draining (dLNs). For each chemokine, the control sample with the lowest expression was used as control and set to 1. Each symbol depicts individual mice among the control and tumor groups. Two-tailed Student's t-test has been used in panel A. *p<0.05 by one-way ANOVA in panel B.

5.5.3. A decreased frequency of B cells has been observed in MCs-deficient tumors

Our data revealed a role of the MC in the regulation of B cells activation both in the physiological and in the pathological contexts of the intestinal inflammation. We were afterwards interested in determining whether MCs could affect B cells in the framework of the CRC. Therefore, we analysed B cells' distribution, phenotypical markers and Ab production comparing Wt, *Kit*$^{W\text{-}sh}$ and rec-*Kit*$^{W\text{-}sh}$

MC-38 tumor-bearing mice. The reconstitution of *Kit*$^{W\text{-}sh}$ was done i.p. before the i.d. injection of MC-38 cells in the mice flanks as described in material and methods (*paragraph 7.2.2)*. We analysed B cells' accumulation as in paragraph 5.4.1, but no relevant differences were observed whether the MC was present or absent. All the three groups of tumor-bearing mice presented a higher frequency of CD19^{+} cells in dLNs compared to ndLNs, meaning that this result was not dependent on the MC (*figure 44A*). Since the MC is reported to be an important infiltrating cell type in the context of CRC[245], we analysed the B cell population infiltrating the tumor mass by flow cytometry (*figure 44A*) and through histological analysis (*figure 44B*). Interestingly, the histological analysis of tumors showed a higher number of infiltrating B cells (CD45R^{+} elements) in Wt tumors and in the ones grown in rec-*Kit*$^{W\text{-}sh}$ mice compared to *Kit*$^{W\text{-}sh}$ tumors (*figure 44B*).

Although no significant difference was instead obtained through flow cytometry between the Wt and *Kit*$^{W\text{-}sh}$ conditions, a higher percentage of B cells was observed in rec- *Kit*$^{W\text{-}sh}$ tumor mice by flow cytometry (*figure 44A*), indicating that the reconstitution of the MC population before the tumor establishment implies a higher infiltration of B cells in the tumor.

We also analysed the expression of membrane activator markers and molecules associated to a tumor phenotype on splenic CD19^{+} cells: FasL resulted to be differently expressed on rec-*Kit*$^{W\text{-}sh}$ splenic B lymphocytes, and a similar trend was detected also for PD-L1 (*figure 45A*). This increased expression suggests that MC reconstitution has a role in the induction of a specific cancer phenotype of splenic B cells. Splenic purified B cells were then tested for their capability to produce Igs. *Ex vivo*, while no differences were observed in the presence or absence of MCs in the levels of released IgG, higher IgM amounts were detected in the absence of MCs from resting and a-CD40 mAb stimulated B cells that returned to normal levels upon MCs reconstitution. On the contrary, splenic B cells from *Kit*$^{W\text{-}sh}$-tumor-bearing mice released higher levels of IgA in the presence or absence of the stimulations. Interestingly, in the rec-*Kit*$^{W\text{-}sh}$ -tumor mice increased quantities of this Ab isotype were detected compared to the MC-deficient tumor condition, suggesting again a role of MCs in promoting B cells activation in tumor mice (*figure 45B*).

Considering altogether the reported differences between the *Kit*$^{W\text{-}sh}$ and the rec-*Kit*$^{W\text{-}sh}$ -tumor mice conditions, we can speculate that in the context of tumorigenesis the activation of MCs directly or indirectly contribute to enhance B cells' activation in tumor-bearing mice.

Tumor mice

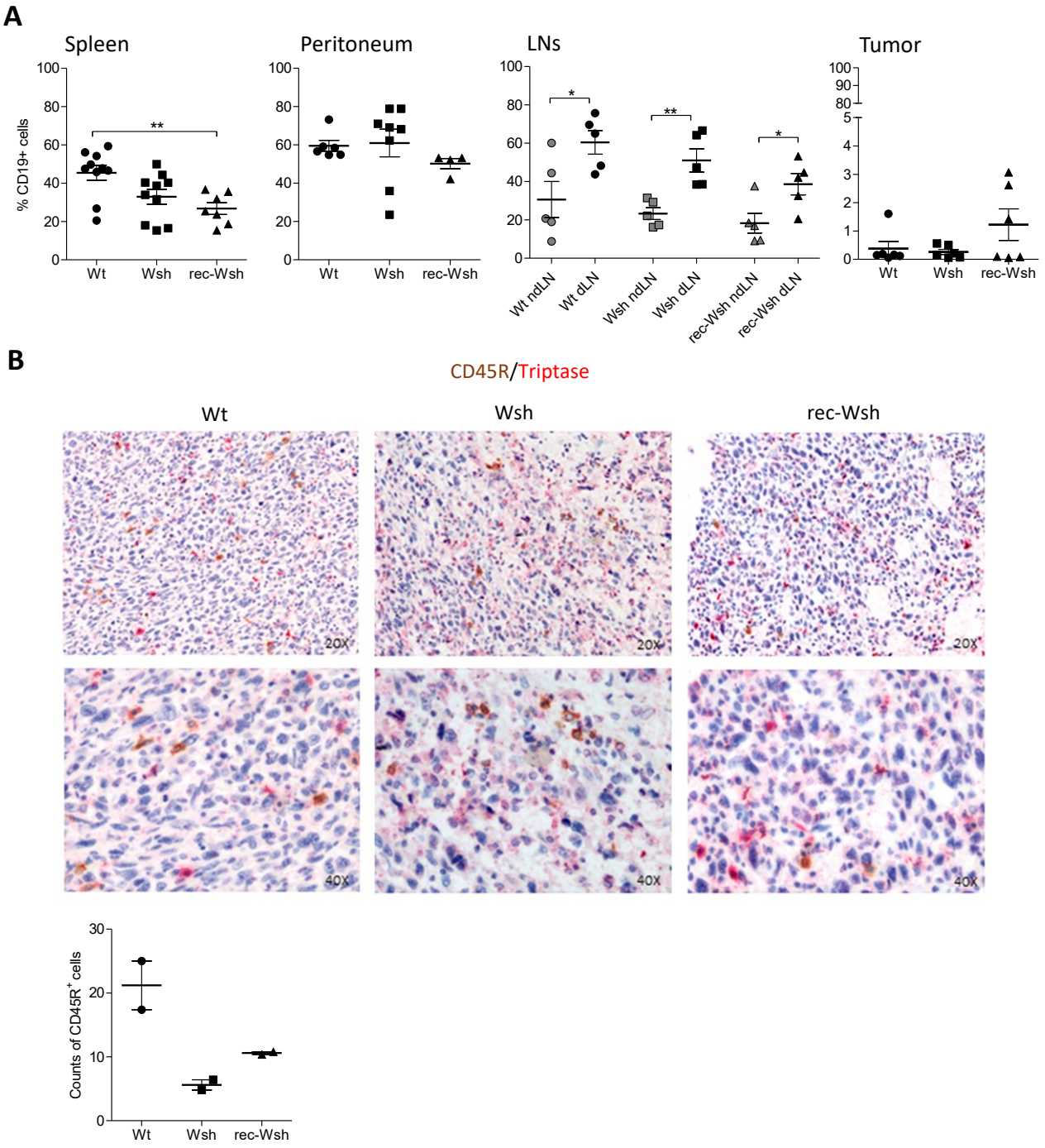


Figure 44: B cells' distribution in Wt, Wsh and rec-Wsh tumor-bearing mice. (A) Percentages of $CD19^+$ cells were analysed in cell suspensions obtained from the spleen, peritoneum and LNs of Wt, Wsh, and rec-Wsh tumor-bearing mice by flow cytometry. B lymphocytes were also identified by flow cytometry after tumor digestion (far right panel). **(B)** Representative immunohistochemistry analysis of anti-CD45R and anti-tryptase immunostaining of formalin-fixed paraffin-embedded sections of MC-38 tumors grown in Wt, Wsh and rec-Wsh mice. Counts of CD45R elements are also provided in the graph below where each symbol of the scatter plot is the mean count of 5 fields (40x).
Each symbol depicts individual mice among the three tumor groups. Analysis were done by two-tailed Student's t-test or by one-way ANOVA, *p<0.05, **p<0.01.

Tumor mice

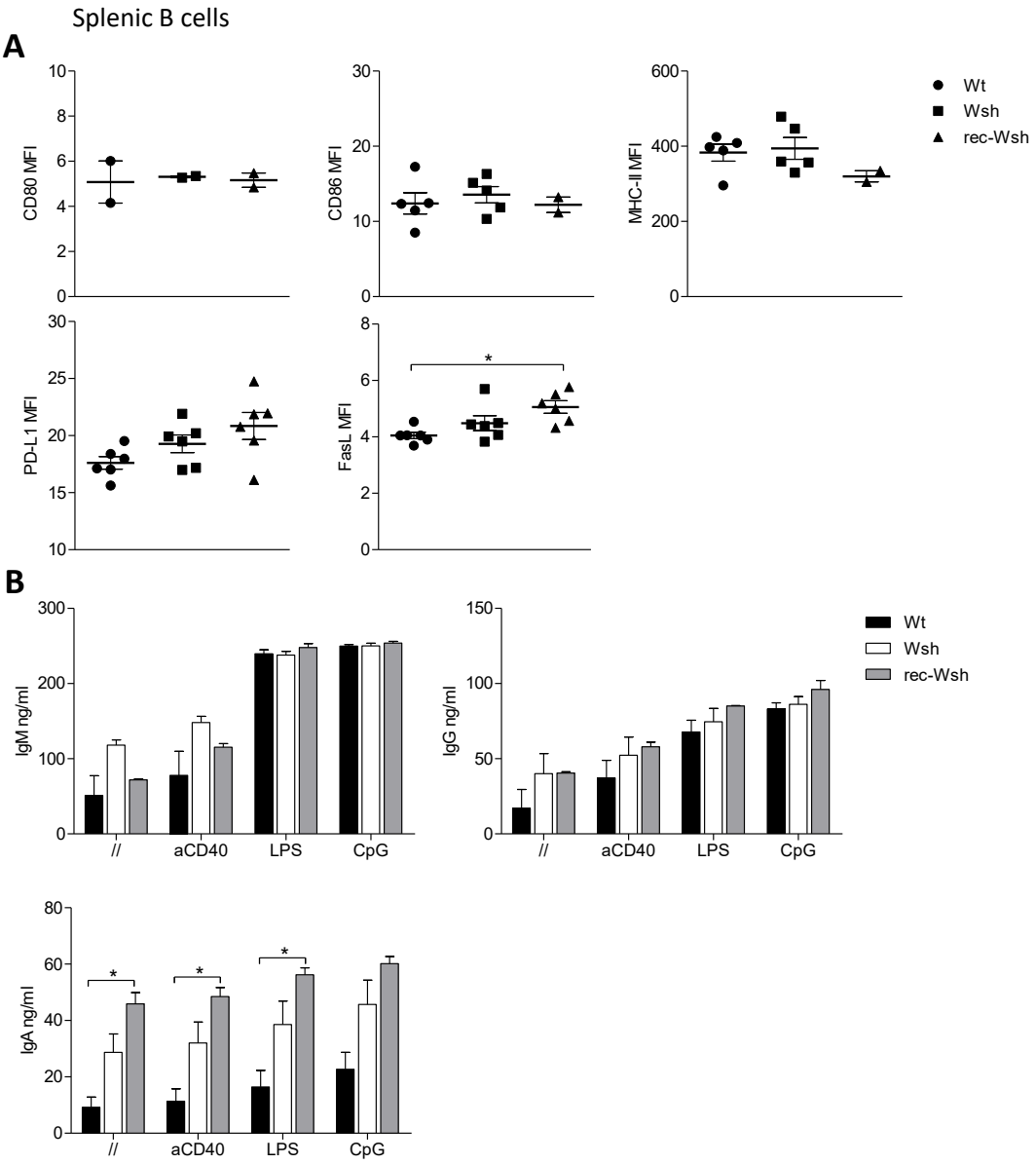


Figure 45: Splenic B cells' phenotype and antibody production comparing Wt, Wsh and rec-Wsh tumor-bearing mice. (A) $CD19^+$ splenocytes from tumor-bearing mice were analysed for membrane expression of CD80, CD86, MHC-II, PD-L1 and FasL and data are indicated as MFI. Each symbol depicts individual mice among the three tumor groups. **(B)** IgM, IgG and IgA were detected by ELISA in supernatants of 48h cultured resting or stimulated (anti-CD40 mAb, LPS, CpG) splenic B cells. Results are the means +SEM from 3 mice in the Wt and Wsh groups and from 2 mice in the rec-Wsh condition. Analyses were done by two-tailed Student's t-test or by one-way ANOVA, *p<0.05

5.5.4. CXCL13 and CCL20 overexpression in dLNs depends on the presence of MCs

Since tumor dLNs are extremely important predictors of cancer spread[305,306], among the observed differences we decided to deeper investigate the accumulation of B cells in tumor dLNs. In order to

do so, we evaluated the expression of chemokine receptors and of the related-chemokines in LNs comparing Wt, *Kit*$^{W\text{-}sh}$, rec- *Kit*$^{W\text{-}sh}$ ndLNs and dLNs. Although no significant differences were detected in the expression of LNs-related B cell chemokine receptors between Wt, *Kit*$^{W\text{-}sh}$ and rec-*Kit*$^{W\text{-}sh}$ tumor mice splenic B cells, a tendency toward an increase in the expression of CXCR5 and CCR6 in W-sh mice was observed (*figure 46A*). Importantly, this effect was reverted in rec- *Kit*$^{W\text{-}sh}$ splenic B cells. Moving to the analysis of chemokine expression in ndLNs and dLNs, a trend toward an increased expression of CCL19 and CXCL13 in Wt dLNs compared to matching ndLNs, and a significant increase in the case of CCL20 were detected. Importantly, in *Kit*$^{W\text{-}sh}$ tumor-bearing mice these differences were abrogated and, remarkably, in MCs-reconstituted tumor-bearing mice the increase in the expression of CXCL13, CCL19 and CCL20 in dLNs was restored (*figure 46B*). These results suggest a role imputable to MCs for the sustained expression of B cell-related chemokines.

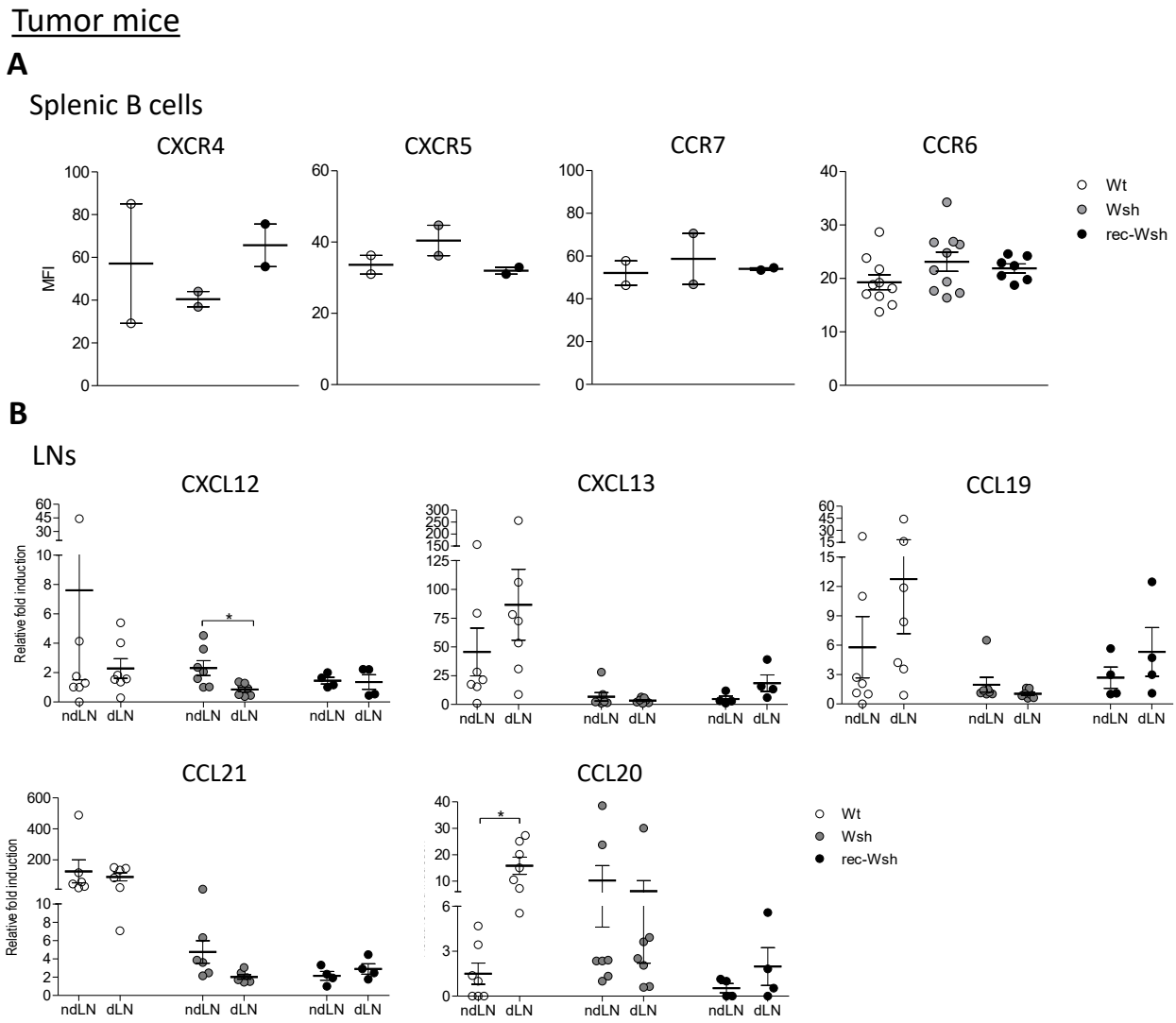


Figure 46: CXCL13 and CCL20 overexpression in dLNs is lost in Wsh tumor mice. (A) Surface expression of CXCR4, CXCR5, CCR7 and CCR6 chemokine receptors was analysed by flow cytometry on CD19^{+} splenocytes isolated from Wt, Wsh and rec-Wsh MC-38 tumor-bearing mice and MFI is indicated in the graphs. **(B)**

Relative expression of cxcl12, cxcl13, ccl19, ccl21 and ccl20, normalized to the housekeeping gene g3pdh, was analysed in the total cellular population of LNs isolated from tumor mice by qPCR. LNs were distinguished between ndLNs and dLNs. For each chemokine, the ndLN sample with the lowest expression within each one of the three groups of mice was used as control and set to one. Each symbol depicts individual mice. One-way ANOVA has been used in panel A, two-tailed Student's t-test has been used in panel B, *p<0.05.

Among the chemokines that were differentially expressed in dLNs (compared to ndLNs) the analysis of the CCL20 chemokine reached statistical significance in the Wt tumor condition. We decided to proceed with a more detailed study of this axis since the involvement of the CCL20/CCR6 axis has been linked to both human and murine CRC by many authors as it is described in the introduction of this thesis and reviewed in[271].

Our aim was to determine whether B cells that accumulate in dLNs had a higher expression of the CCL20 receptor as a consequence of its ligand enhanced gradient. From our analysis, we observed that B cells that home dLNs have an increased expression of CCR6 receptor in all the three models of tumor-bearing mice. Although we proposed that at the peripheral level splenic B cells have the same ability to migrate towards CCL20 gradients, a real implication of CCL20 in promoting B cell accumulation in dLNs can be hypothesized. Moreover, interestingly, in MCs-reconstituted mice, the expression of CCR6 on dLNs B cells was even higher compared the tumor Wt B cell condition (*figure 47*).

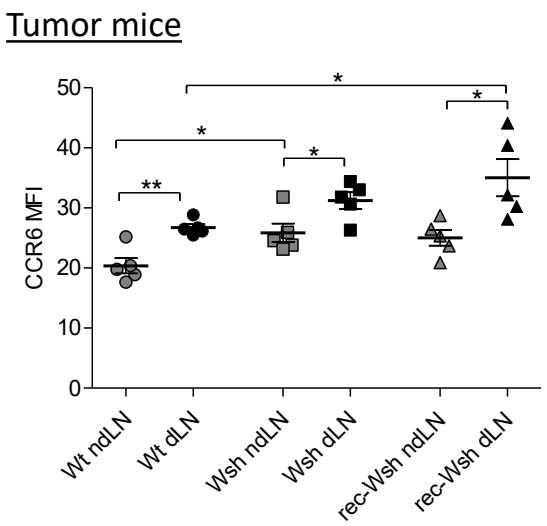


Figure 47: B cells that accumulated in tumor-draining LNs had a higher expression of CCR6. $CD19^+$ lymphocytes in cell suspension from Wt, Wsh and rec-Wsh tumor nd- and dLNs were analysed for the membrane expression of CCR6 by flow cytometry.
Each symbol depicts individual mice among the tumor groups. Analyses are done by two-tailed Student's t-test (ndLNs versus dLNs within each mice genotype) or by one-way ANOVA between ndLNs or dLNs of the 3 groups, *p<0.05, **p<0.01.

5.5.5. Serum concentrations of CCL20 are increased after tumor development in Wt but not in W-sh mice

Our data suggest a role for CCL20 and CXCL13 in the accumulation of B cells in the tumor dLNs and interestingly, CCL20 has been proposed to be a prognostic factor in CRC since its serum levels increase during its development[307]. Moreover the axis CXCL13/CXCR5 has also been associated to CRC progression and metastasis[308].

With the purpose of determining if in our tumor model a systemic increase of these two chemokines was present, we measured their concentrations in sera samples comparing levels before the injection of the tumor and at the moment of the sacrifice. Our results showed a significant increase of CCL20 and a trend towards increased levels of CXCL13 upon tumor development in Wt mice. In *Kit*$^{W\text{-}sh}$ MC38-tumor mice these increases were not maintained after the development of the tumors. To date, due to the lack of an appropriate number of mice in the analysis of the rec-*Kit*$^{W\text{-}sh}$ tumor condition, we are not able to ascribe to the absence of MCs the systemic effect of chemokines up-regulation *(figure 48)*.

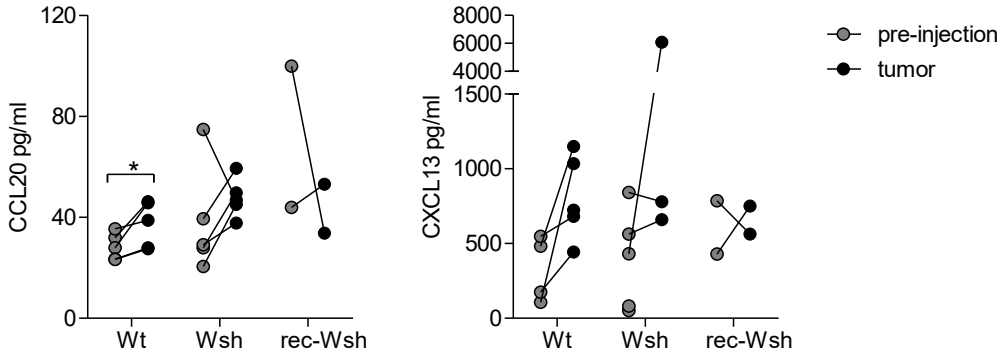


Figure 48: CCL20 is increased in the serum of MC-38-tumor-bearing Wt mice. CCL20 and CXCL13 concentrations were measured by ELISA from Wt, Wsh and re-Wsh mice blood samples collected before the injection of the M-C38 cell line (pre-injection) and at the moment of the sacrifice (tumor). Each symbol depicts individual mice. The lines follow the trend of the CCL20 serum concentration of a single mouse. Analyses are done by two-tailed Student's t-test within each one of the three mice groups, *p<0.05.

5.6. IS THE DIRECT MC-CANCER CELL INTERACTION ABLE TO CREATE FAVORABLE MICRO- AND MACRO-ENVIRONMENTS FOR B CELL ACTIVATION AND CHEMOTAXIS?

With the last part of this thesis we tried to clarify whether some traits of MCs' phenotype and behaviour in the CRC setting were responsible for promoting B cells' accumulation in the anatomical sites affected by the tumor. For our analysis we assumed that MCs establish a cross-talk with cancer cells since they are known to be recruited and to proliferate in the tumor. Furthermore we considered that MC's biology in malignancies is characterized by plasticity and heterogeneity in phenotypes and functions[309,310]. We first determined whether the MCs/CRC cells direct interaction induced a modulation in the expression of MCs' membrane-bound receptors, next we analysed the release of soluble mediators. These two parameters were accounted for defining a specific cancer phenotype of MCs. Besides, we evaluated the modulation in the expression of chemokines that could be involved in the accumulation of B cells in both MCs and cancer cells.

5.6.1. Surface markers expression on MCs are not modulated in co-culture with cancer cells

To determine the variations in the membrane phenotype of MCs we established a co-culture between BMMCs and the MC-38 cell line or tumor cells isolated directly from the mass and cultured *ex vivo* (for further details about the procedure, see *section 7.2.8*).

After 24h of co-culture, MCs were analysed by flow cytometry for the expression of inflammatory receptors (ST2, TLR2 and TLR4), the activatory molecule MHC-II, the adhesion molecules ICAM-1 and co-stimulatory molecules (CD40L, OX40L, CD30L and CD28). We chose to explore this set of receptors because of their importance in MCs' activation, while co-stimulatory molecules were analyzed for their importance in the interplay with other leukocytes infiltrating the tumor, such as B cells. In *figure 49* it is possible to observe that no differences in the expression of these molecules were identified after MCs' culture in the presence of MC-38 cells or *ex vivo* cultured tumor cells.

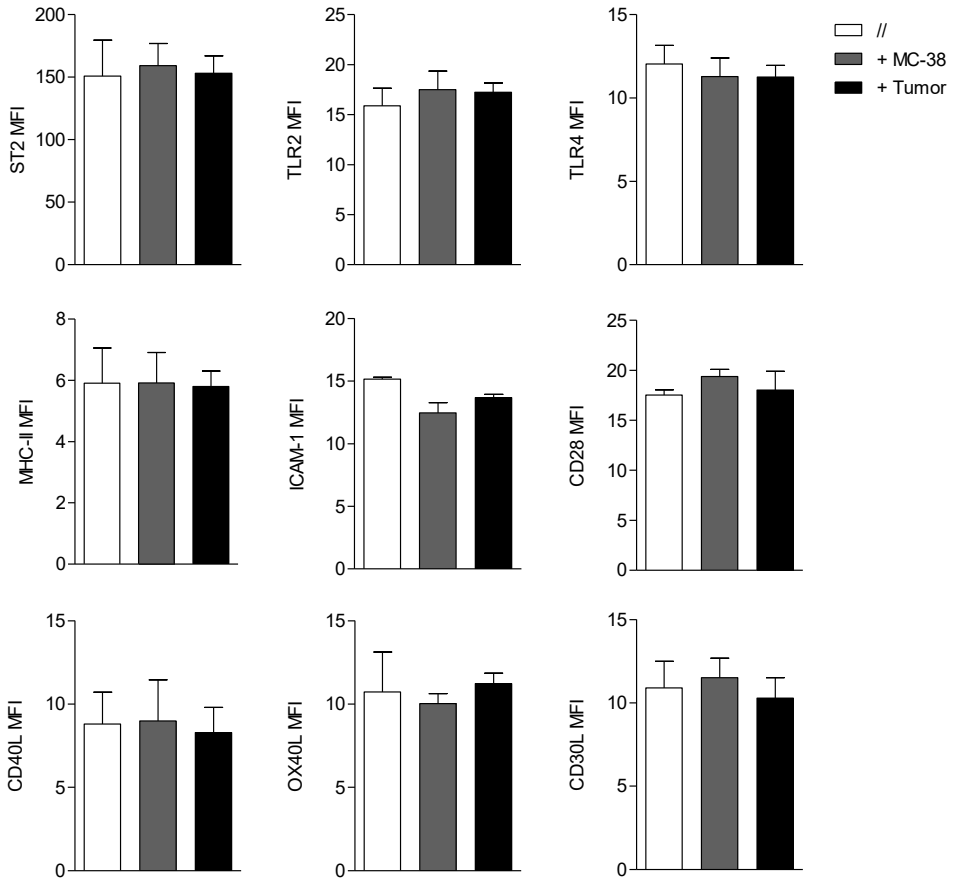


Figure 49: Cancer cells do not affect the expression of MCs' surface markers. Surface expression of ST2, TLR2, TLR4, MHC-II, ICAM-I, CD28, CD40L, OX40L and CD30L was analysed by flow cytometry on c-Kit$^+$ BMMCs alone (//), after 24h of co-culture with the MC-38 cell line (+MC-38) or with MC-38 tumor-derived adherent cells (+ Tumor). MFI are indicated in the graphs. The results reported are mean (+ SEM) of 3 independent experiments. There were no statistically significant differences between group means as determined by one-way ANOVA.

5.6.2. MCs release soluble mediators during direct cross-talk with cancer cells

Since the analysis of this set of membrane molecules did not result in cancer-driven phenotype for MCs, we wondered whether MCs-derived soluble mediators could be involved in the modulation of the tumor microenvironment (TME)[248]. In order to mimic the TME we set up our co-cultures in the absence or in the presence of the IL-33, alternatively. The importance of IL-33 during CRC progression, even if debated, has been documented and the dynamics of the axis ST2/IL-33 have been thoroughly studied. In fact its increased activation along the colorectal adenoma–carcinoma sequence is involved in the neoplastic transformation[311]. Moreover, MCs, which are a target of therapies in many solid tumors, are highly sensitive to the IL-33 cytokine[96].

Supernatants from co-culture between BMMCs and MC-38 cells or *ex vivo* adherent tumor cells were tested for TNF-α, IL-6 and IL-13, since these cytokine are usually released by activated MCs

and can participate in creating a pro-inflammatory environment[312]. In addition we quantified also IL-10, which generally promotes tumor growth, and SCF, that is a factor released by tumor cells involved in the recruitment and the activation of MCs during tumor development[247].

No TNF-α release was observed from BMMCs and MC-38 cells cultured alone in resting conditions, while the mediator was detectable after 1h, increased at 5h and still detectable after 24h, in the co-culture (*figure 50A*). The addition of IL-33 in the co-culture medium stimulated TNF-α release by BMMCs cultured alone, while MC-38 cells do not produce this factor after IL-33 stimulation (lower graphs in *figure 50A*). Interestingly, also in this case, in the co-culture, higher amounts of TNF-α were measured compared to the BMMCs condition alone, suggesting that the contact with MC-38 cells promoted a spontaneous release of this pro-inflammatory factor from MCs. Moreover, by establishing the co-culture between MCs and *ex vivo* derived-tumor cells we were able to reproduce the same effect (*figure 50B*).

To confirm that MCs were responsible for the TNF-α release in the co-culture, by taking advantage of TNF-α K.O. BMMCs, we set up the same co-culture system at 1 and 5h time points and no mediator release was detectable. Hence we confirmed that MCs were the source of the TNF-α early released just after the encounter with CRC cells without the presence of any other stimulation (*figure 50C*).

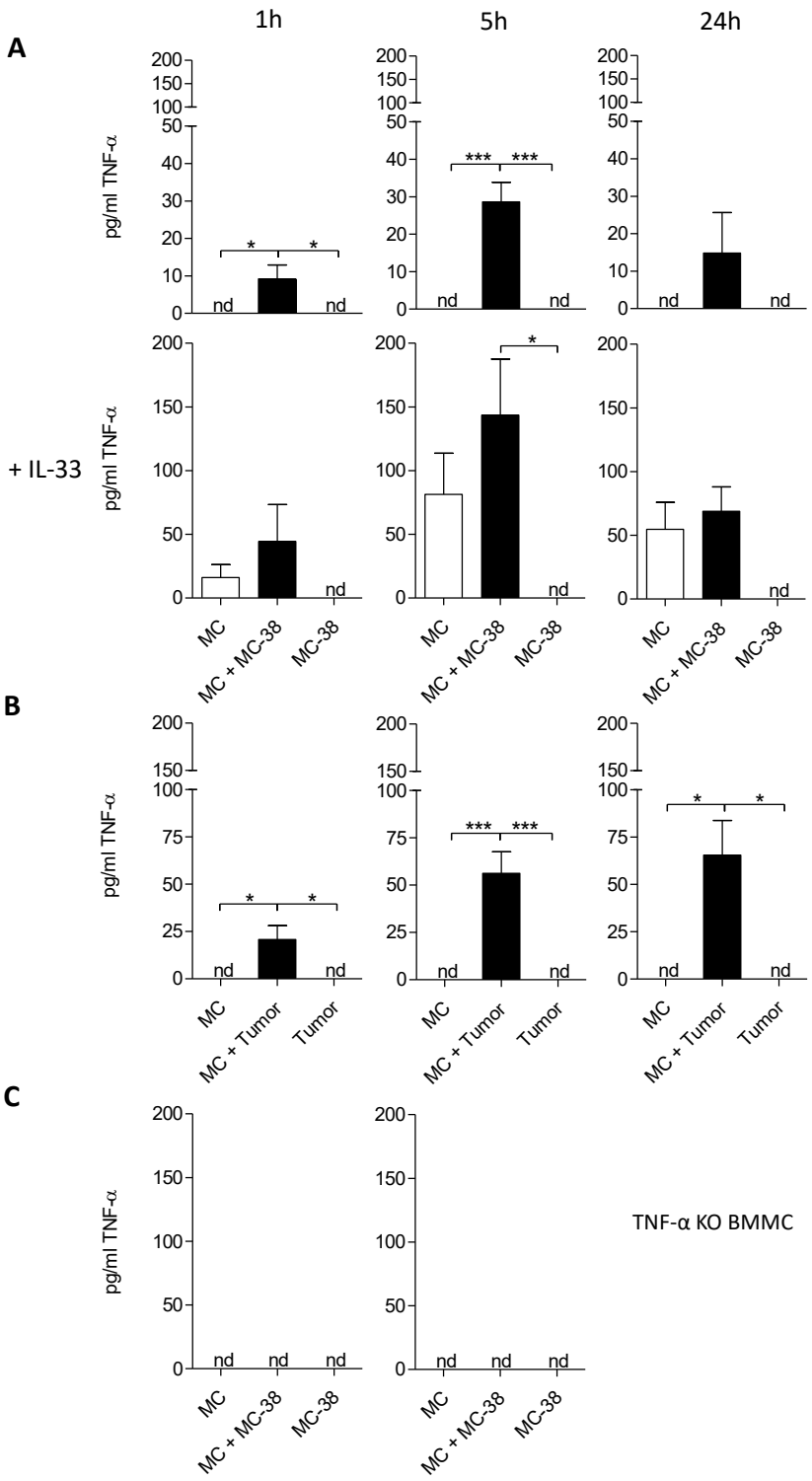


Figure 50: The contact between MCs and CRC cells promoted the release of the pro-inflammatory TNF-α factor by MCs. TNF- α was measured by ELISA in 1, 5 and 24h supernatants derived from:
(A) BMMCs (MC) and MC-38 cell line cultured alone or in co-culture. In the lower graphs, 50 ng/ml of IL-33 was added to the culture medium.
(B) BMMCs cultured alone or with adherent cells derived from MC-38 tumor mass grown in C57BL/6 mice (Tumor).
(C) BMMCs TNF-α K.O. cultured alone or in the presence of MC-38 cells.
The results reported are mean (+ SEM) from at least 3 independent experiments, *p<0.05, ***p<0.001 by one-way ANOVA. nd = not detected

We then measured IL-6, IL-13 and IL-10 cytokines, adding the 48h time point since we cannot exclude late production of these de novo synthesized mediators by MCs.

Similar results to TNF-α were obtained for IL-6: no IL-6 has been detected from BMMCs, MC-38 cells or tumor cells cultured alone, while in the co-culture IL-6 levels in the supernatants increased (*figure 51A* and *B*). Previous results were retrieved also upon IL-33 stimulation: BMMCs (and not MC-38

cells) release IL-6 after the addition of the cytokine, but, in the co-culture, the release is higher, indicating that cancer cells are able to increase the production of these cytokine.

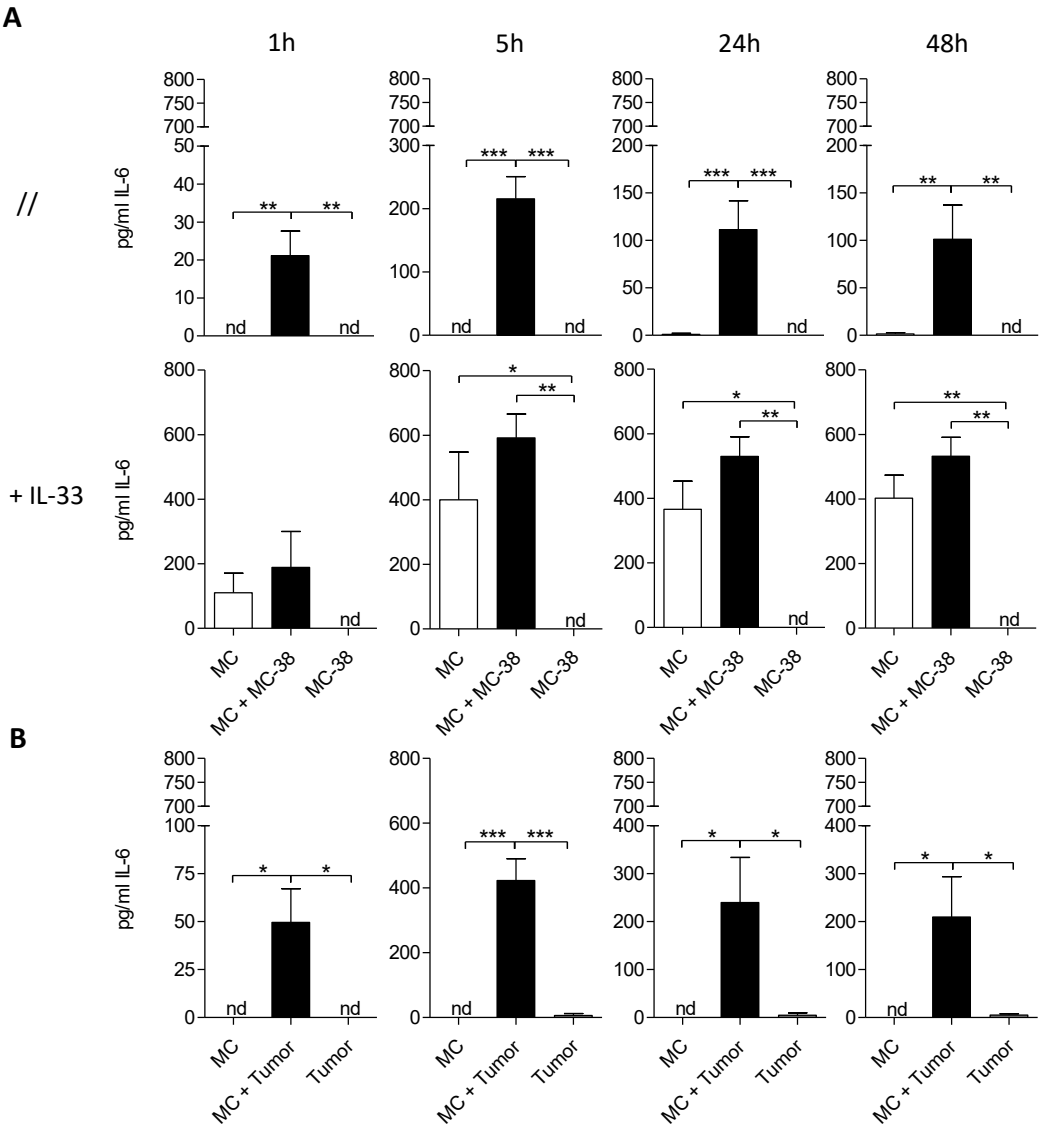


Figure 51: The contact between MCs and CRC cells promoted the release of the pro-inflammatory IL-6.
IL- 6 was measured by ELISA in 1, 5, 24 and 48h supernatants derived from:
(A) BMMCs (MC) and MC-38 cell line cultured alone or in co-culture. In the lower charts, 50 ng/ml of IL-33 was added to the culture medium.
(B) BMMCs cultured alone or with adherent cells derived from MC-38 tumor mass grown in C57BL/6 mice (Tumor).
The results reported are mean (+ SEM) from at least 3 independent experiments, *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA. nd = not detected

To elucidate better this second evidence, we performed qPCR analyses for il-6 mRNA expression on BMMCs and MC-38 cells cultured for 5h alone or in co-culture. As shown in *figure 52A*, only MC-38 cells start transcribing *il-6* gene post-co-culture with BMMCs (and not *vice versa*). Moreover, an ICS analysis for the IL-6 protein was performed on MC-38 cells after culturing the cells o.n. with BMMCs

in the presence of monensin. As shown in a representative experiment depicted in *figure 52B*, the ICS result confirms the qPCR analysis, showing an increased production of IL-6 (from the 6% of the basal level to the 12% of the co-culture) after the co-culture with MCs. It is therefore probable that the encounter between MCs and cancer cells favoured the establishment of an IL-6 enriched microenvironment where MCs contributed with an early release of the mediator (similarly to what observed with TNF-α) and tumor cells participate with a late production sustained by the presence of MCs.

Similar considerations can be made concerning IL-13 production in the co-culture system (*figure 53*), it is possible that MCs and cancer cells sustain each other in the production of the mediator. IL-13 can therefore be included among the mediators that create a pro-inflammatory milieu during MC/CRC interaction.

IL-10, an anti-inflammatory mediator that is known to favour tumor progression, was on the contrary not released in MC-cancer cell interaction (data not shown). This result corroborates the specificity of the establishment of a pro-inflammatory TME, consequence of the accumulation and activation of MCs at tumor sites.

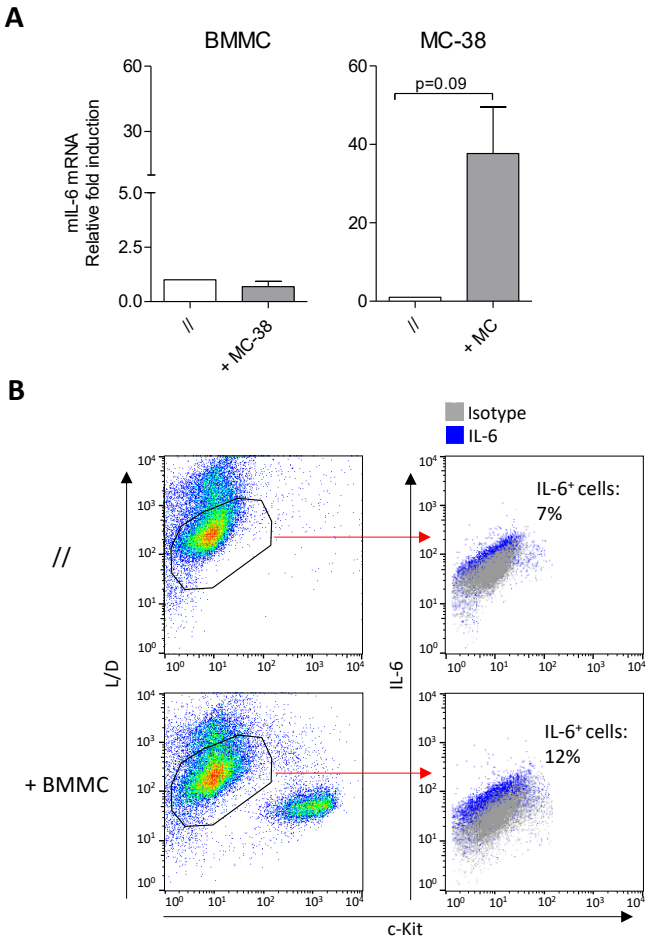


Figure 52: MC-38 cells increased their production of IL-6 post co-culture with MCs. **(A)** *il-6 gene* expression was analyzed by RT-qPCR after 5h of co-culture between BMMCs and MC-38 cells in normal culture medium. The panel on the left shows il-6 expression in BMMCs that were cultured alone (//) or with MC-38 cells, the right panel shows il-6 expression in MC-38 cells cultured alone (//) or with BMMCs (+ MC). Relative fold induction over BMMCs (on the left) and MC-38 (on the right) are indicated. Means (+SEM) of three independent experiments analysed by two-tailed Student's t-test are shown in the graphs. **(B)** Representative ICS for IL-6 performed in MC-38 cells (//) and MC-38 + BMMCs (upper and lower density plots respectively). Cells, gated on their double negativity for the Live/Dead and c-Kit staining (left panels) are identified as % of IL-6$^+$ cells based on respective control isotype staining (right panels).

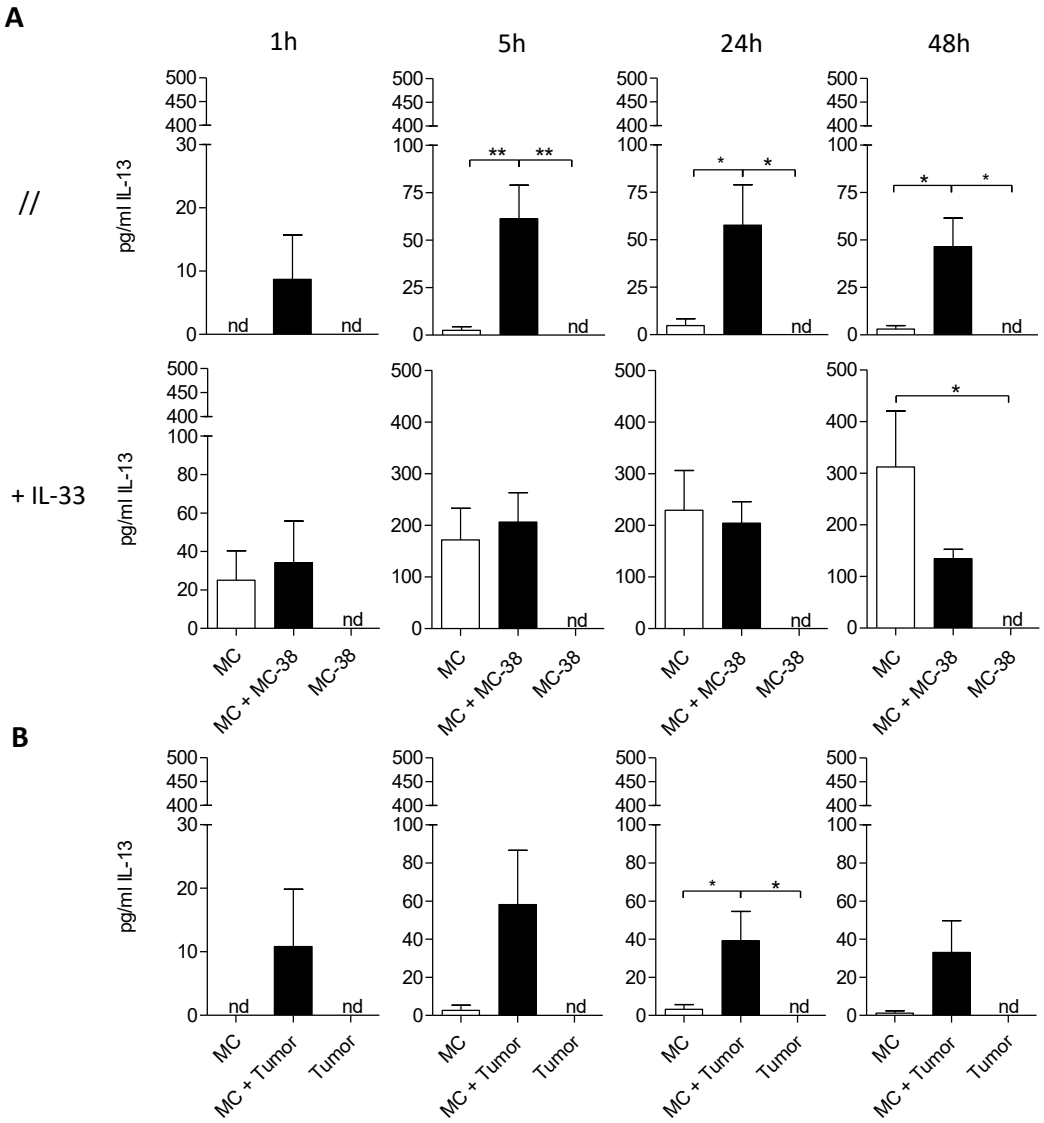


Figure 53: The contact between MCs and CRC cells promotes the release of IL-13. IL- 13 was measured by ELISA in 1, 5, 24 and 48h supernatants derived from:
(A) BMMCs (MC) and MC-38 cell line cultured alone or in co-culture. In the lower graphs, 50ng/ml of IL-33 was added to the culture medium.
(B) BMMCs cultured alone or with adherent cells derived from MC-38 tumor mass grown in C57BL/6 mice (Tumor).
The results reported are mean (+ SEM) from at least 3 independent experiments, *p<0.05, **p<0.01, by one-way ANOVA. nd = not detected.

5.6.3. Soluble factors derived from MC-38 cells are sufficient to induce the release of pro-inflammatory mediators by MCs

Taken together, our data indicate that MCs are induced by tumor cells and by the tumor context (mimed by adding IL-33 in the co-culture system) to release pro-inflammatory cytokines. In order to determine whether this is a contact-dependent mechanism or it is driven by soluble factors released

by tumor cells, we stimulated MCs with an 8h conditioned medium derived from MC-38 cells and analyzed TNF-α and IL-6 release after 1 and 5h. Interestingly, as shown in *figure 54*, the MC-38-derived conditioned medium is sufficient to promote the release of both the soluble factors analyzed by MCs, the effect was even stronger than the one observed in the co-culture of the two cell types.

SCF is an important factor released from tumor cells that can strongly activate MCs' tumor infiltration and production of mediators[247]. In this regard, we decided to test in our system the possibility that MCs are induced to release the factors observed because of MC-38 cells' production of SCF. As shown in *figure 55*, MC-38 cells release SCF without stimulation, while in the presence of MCs it is probably consumed. In the presence of IL-33, SCF production is marginally impacted.

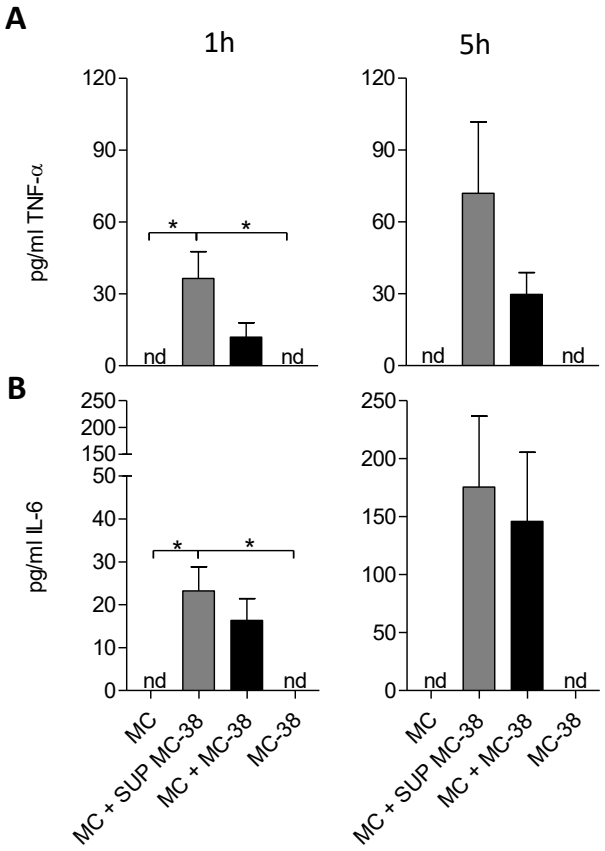


Figure 54: The MC-38 cells' derived supernatant is sufficient to induce MC's release of TNF-α and IL-6. TNF-α **(A)** and IL-6 **(B)** were measured by ELISA in 1 and 5h supernatants from BMMCs (MC) and MC-38 cell line cultured alone or in co-culture and from BMMCs cultured in the 8h conditioned medium derived from MC-38 cells (MC + SUP MC-38).
The results reported are means (+ SEM) from 3 independent experiments. *p<0.05 by one-way ANOVA. nd = not detected.

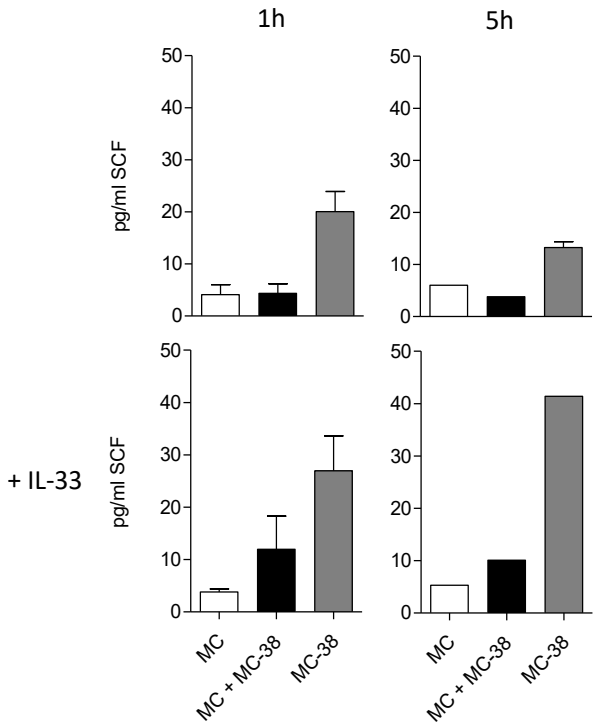


Figure 55: SCF is detected in the MC/MC-38 co-culture system. SCF was measured by ELISA in 1 and 5h supernatants derived from BMMCs (MC) and MC-38 cell line cultured alone or in co-culture. In lower graphs, 50 ng/ml of IL-33 was added in the culture medium.
Reported results are mean (+ SEM) from 2 and 1 experiments respectively at 1 and 5h time points.

5.6.4. The release of soluble mediators by MCs is specifically induced by CRC cell lines

We were then interested in determining whether or not the effects observed in the release of pro-inflammatory mediators by MCs were specific of the cross-talk with CRC cell lines. To address this question we established control co-cultures between BMMCs with CT26 (a fibroblast colon carcinoma cell line), B16-FO melanoma and 4T1 breast cancer cell lines, matching the BALB/c or C57BL/6 background. Similarly to what observed with MC-38, by using CT26 colon carcinoma cell line, IL-6 was released. Moreover we observed not detectable levels of TNF-α and no release of IL-10. By culturing MCs with B16 melanoma cells we found no release of TNF-α and IL-6, and detectable levels of SCF, even though with a reduced concentration compared to MC-38 cells (*figures 56 and 55*). Finally, by using 4T1 breast cancer cell line, we observed no release of TNF-α in the co-culture system and low levels of IL-6 (less than 10 pg/ml) compared to the 200 pg/ml measured in the co-culture with MC-38 and the 50 pg/ml of the CT26. To sum up, the effects we observed in the CRC experimental set up were not reproduced in the context of melanoma and breast cancers.

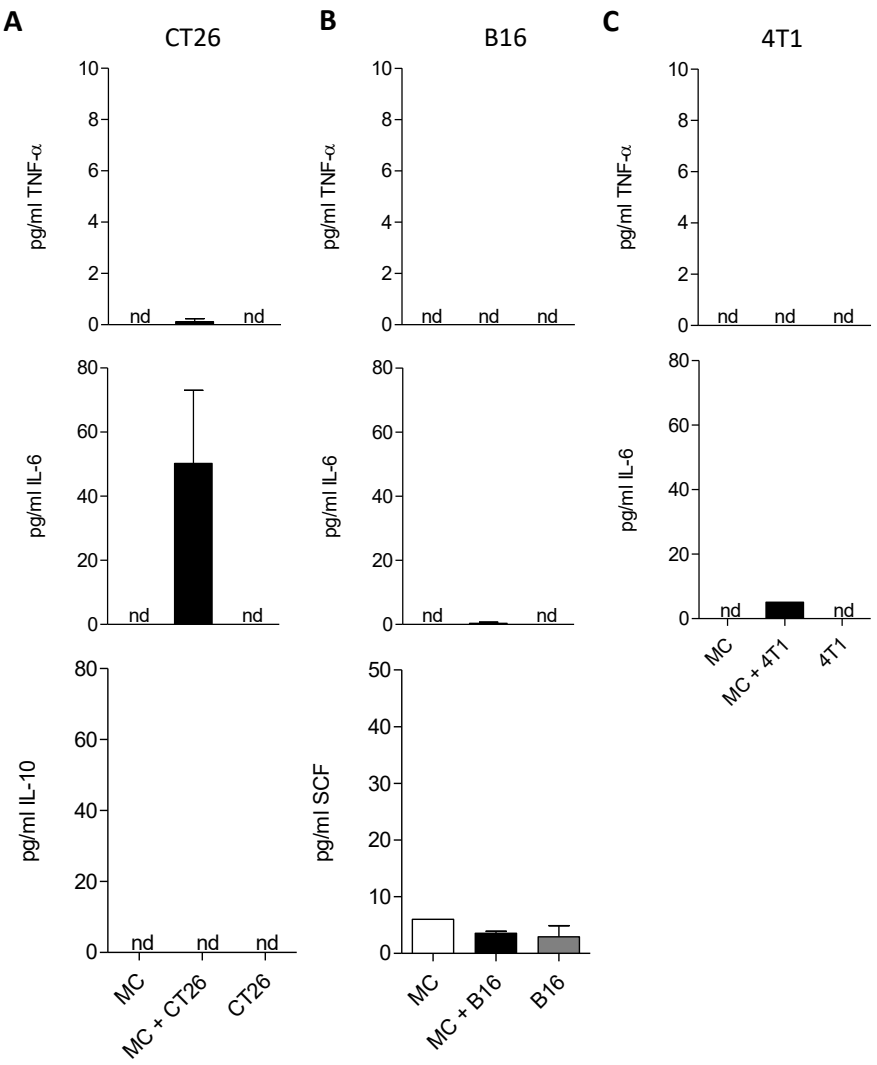


Figure 56: The release of pro-inflammatory mediators is specific for CRC/MC interaction. TNF-α , IL-6 , IL-10 and SCF were measured by ELISA in 5h supernatants derived from BMMCs (MC) and CT26 **(A),** B16 **(B)** and 4T1 **(C)** cell lines culture alone or in co-culture. The results reported are means (+ SEM) from at least 2 independent experiments. nd = not detected.

5.6.5. MCs-cancer cells reciprocal influence in the expression of B-cell related chemokines: the importance of CCL20

The results presented above suggest that infiltrating MCs may alter the TME by influencing cancer cells behaviour and regulating inflammatory and immune reactions. The main purpose of the following experiments is to assess whether the MC-cancer cell interaction can affect B cells' chemotaxis in tumor-bearing mice, as suggested from our *in vivo* data. Therefore, we performed RT-qPCR analyses aimed at investigating the modulation of B cell-related chemokines in the co-culture system between MCs and MC-38 cells. We analysed the transcriptional expression of chemokines in MCs and MC-38 cells in resting conditions and after the conditioning of the presence of the other

cell type in the co-culture system, where a pro-inflammatory environment is established at early time points (1 and 5h) and maintained at longer time points (24 and 48h).

The CCL2 and CCL5 pro-inflammatory chemokines promote the chemotaxis of immune cell types in CRC environment[275] and both are expressed by MCs and MC-38 cells. Increased CCL2 expression in MC-38 was observed post co-culture with MCs, this effect is enhanced in the presence of IL-33 (*figure 57*). CCL5 expression was increased in both MCs and MC-38 cells after the conditioning of the corresponding cell type. Again, in the presence of IL-33, the up-regulation was further increased in the system. It is interesting to highlight that in MCs pre-conditioned by MC-38 cells, a strong upregulation of CCL5 was observed (40 times fold with respect to resting MCs).

Since MC-38 cells are intestinal epithelial cancer cells, we decided to investigate in our system also the modulation of CCL25, CCL28 and CCL20 that contribute to the homing of PCs in the gut[180]. MCs express only CCL25 but its expression is not modulated by the presence of MC-38 cells. Our i*n vitro* cultured MC-38 cells instead expressed CCL25 and CCL20 (and not CCL28, data not shown). While we observed no modulation of CCL25, the expression of CCL20, which was also increased in tumor dLNs (*figure 43 and 46*), is up-regulated in MC-38 cells that were cultured with MCs. Interestingly, the increase in CCL20 transcription is observed again in this context.

We then investigated the expression of CXCL12, CXCL13, CCL19 chemokines in the MC-CRC cross-talk, the other chemokines were analysed in LNs of control and tumor-bearing mice. MCs do not express any of these chemokines (data not shown) while MC-38 cells express detectable levels of CXCL13. However no modulation was observed in the presence of MCs.

It is important to remember that the pattern of chemokines we analysed are B cell-related but not specific, since many immune cell types such as T lymphocytes, monocytes and tumor cells themselves are responsive to their chemotactic action[275].

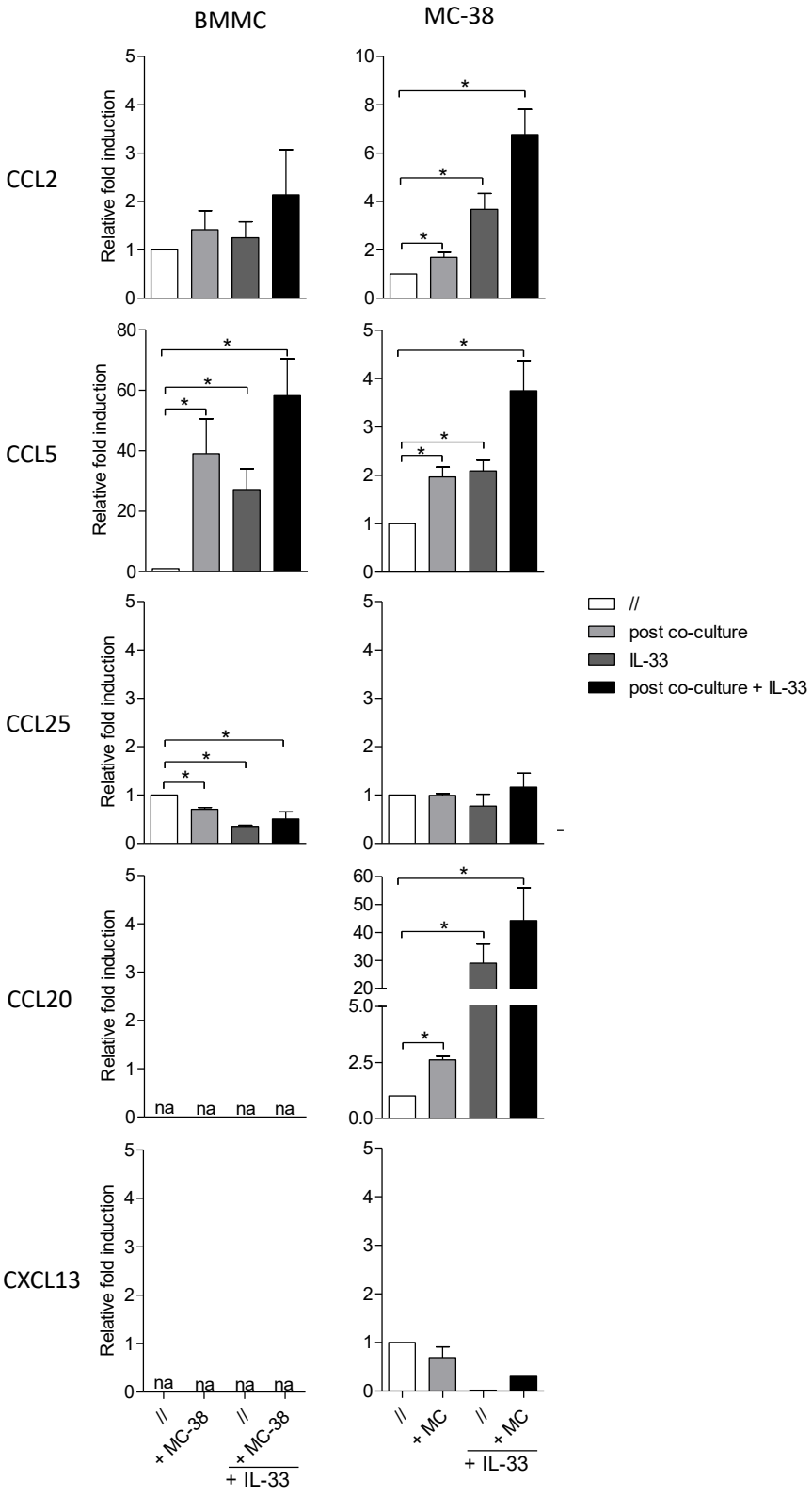


Figure 57: Chemokine expression was modulated in both MCs and MC-38 cells when they cross-talk. *Ccl2, ccl5, ccl25 ccl20* and *cxcl13* gene expression was analyzed by RT-qPCR after 5h of co-culture between BMMCs and MC-38 cells in normal culture medium of in the presence of 50 ng/ml IL-33 (where indicated). Means (+SEM) of at least three independent experiments are shown in the graphs. *p<0.05 by two-tailed Student's t-test. na = not amplified.

5.6.6. TNF-α released by MCs is an essential factor that induces CCL20 expression in MC-38 cells

Our data from MC-38 tumor-bearing mice suggested that CCL20 and CXCL13 play a role in promoting B cells accumulation in dLNs of tumor-bearing mice (*figure 43*). Since in dLNs of *Kit*$^{W\text{-}sh}$ tumor-bearing mice CCL20 and CXCL13 up-regulation was not observed, and neither their seric levels (*figure 48*), we hypothesized that MCs have a role in the induction of these factors. Moreover, analyses conducted in *Kit*$^{W\text{-}sh}$ tumor mice showed reduced levels of TIBs (*figure 44*). It is now evident that MCs promote CCL20 transcriptional expression in MC-38 cancer cells (*figure 57*), this led us to believe that MCs participate in the creation of a CCL20 enriched TME. Accordingly, we decided to study in deep the CCL20 transcriptional and protein modulation in MC-38 cells.

To confirm the specificity of the observed effect in the CRC setting, we analysed the modulation of CCL20 in the presence of BMMCs in CT26, B16 and 4T1 cell lines by qPCR. Interestingly, in CT26 cells CCL20 upregulation after co-culture with MCs has been reproduced. While B16 melanoma cells do not express CCL20, 4T1 cell line expressed CCL20[313] but, interestingly, its level were not modulated in the presence of MCs (*figure 58A*).

We then considered the factors that from the literature are known to be involved in CCL20 transcriptional overexpression: TNF-α, IL-1 and IL-17[314]. We focused our attention on TNF-α since we uncovered that MCs are induced to release this mediator upon stimulation by CRC cells. We set up co-cultures between MC-38 cells and Wt of TNF-α K.O. BMMCs and analysed CCL20 modulation. When culturing the MC-38 cell line with TNF-α K.O. BMMCs, no up-regulation of CCL20 was observed. As a control we also performed an experiment by using IL-6 KO BMMCs. In this case CCL20 was still up-regulated, suggesting that IL-6 is not indispensable for CCL20 transcription, while TNF-α on the contrary is necessary (*figure 58B*). We then wanted to demonstrate the veracity of the TNF-α/CCL20 axis in the MCs/CRC cells at the protein level.

We first performed ELISA measurements for CCL20 in the co-cultures supernatants between MCs with MC-38 cells (in the presence or absence of IL-33), or *ex vivo* MC-38 tumor cells, CT26 and 4T1 cells after 24 and 48h. At 24h time point, the levels of CCL20 released by MC-38 and CT26 alone cultured cells are similar with the ones of their co-cultures with MCs. Differently, at the same 24h time point, a lower extent of CCL20 is shown in the MCs/4T1 co-culture medium compared to 4T1 cells alone. This last result suggests that no support is given by MCs in CCL20 expression (*figure 59*). At 48h, lower levels are detected in all co-culture settings. We can speculate that MCs, upon contact

with cancer cells release pro-inflammatory cytokines but also proteases that could be responsible for chemokine degradation and consumption in the medium. We then analysed soluble CCL20 by comparing co-cultures between Wt or TNF-α K.O. BMMCs and MC-38 cell line (*figure 60*). In these analyses, after 24h of culture, CCL20 concentration is of the same extent between MC-38 alone and in co-culture with Wt BMMCs, while it is inferior in co-culture with TNF-α K.O. MCs, probably because of the lack of the mediator that is fundamental for its increased production.

Further analyses are needed to determine the relevance of MCs' derived TNF-α upon tumor establishment in inducing CCL20 chemokine production by CRC cells and LNs, and the potential role of this axis in the chemotaxis of B lymphocytes in dLNs and tumor. In the conclusive part of this thesis the perspectives of the study are discussed.

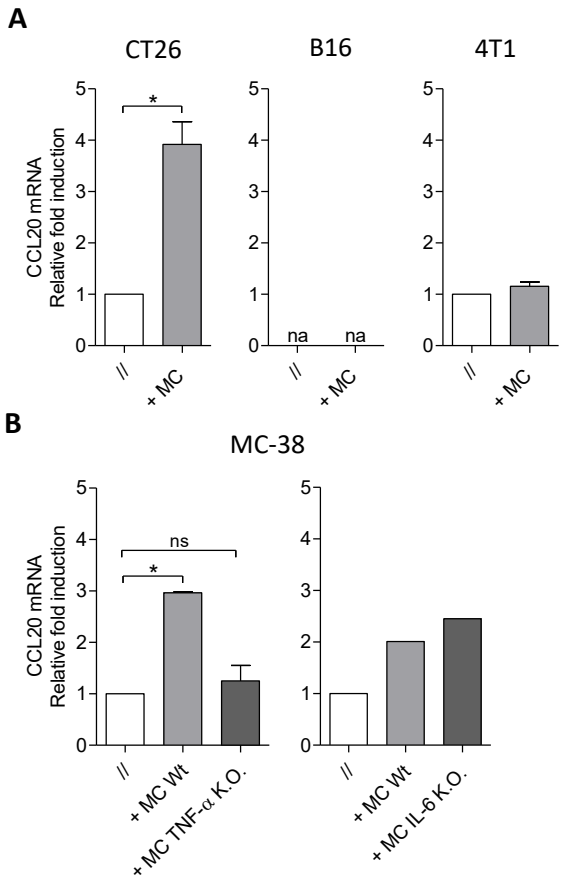


Figure 58: TNF-α derived from MCs is necessary for CCL20 overexpression in CRC tumor cells. (A) *Ccl20* gene expression was analyzed in CT26, B16 or 4T1 cell lines by qPCR after 5h of culture alone (//) or in co-culture with BMMCs (+ MC). **(B)** *Ccl20* gene expression analyzed in MC-38 cells that were cultured alone or with BMMCs Wt or TNF-α K.O. (left panel) and with Wt or IL-6 K.O. BMMCs (right panel).

Relative fold induction over each cell line cultured alone are indicated. Mean (+SEM) from two independent experiments is shown for 4T1 cells and a representative experiment in shown in the comparison between co-culture with Wt or IL-6 K.O. cells; other graphs are mean (+SEM) of at least three independent experiments. *p<0.05 by two-tailed Student's t-test.

na = not amplified, ns = not significant.

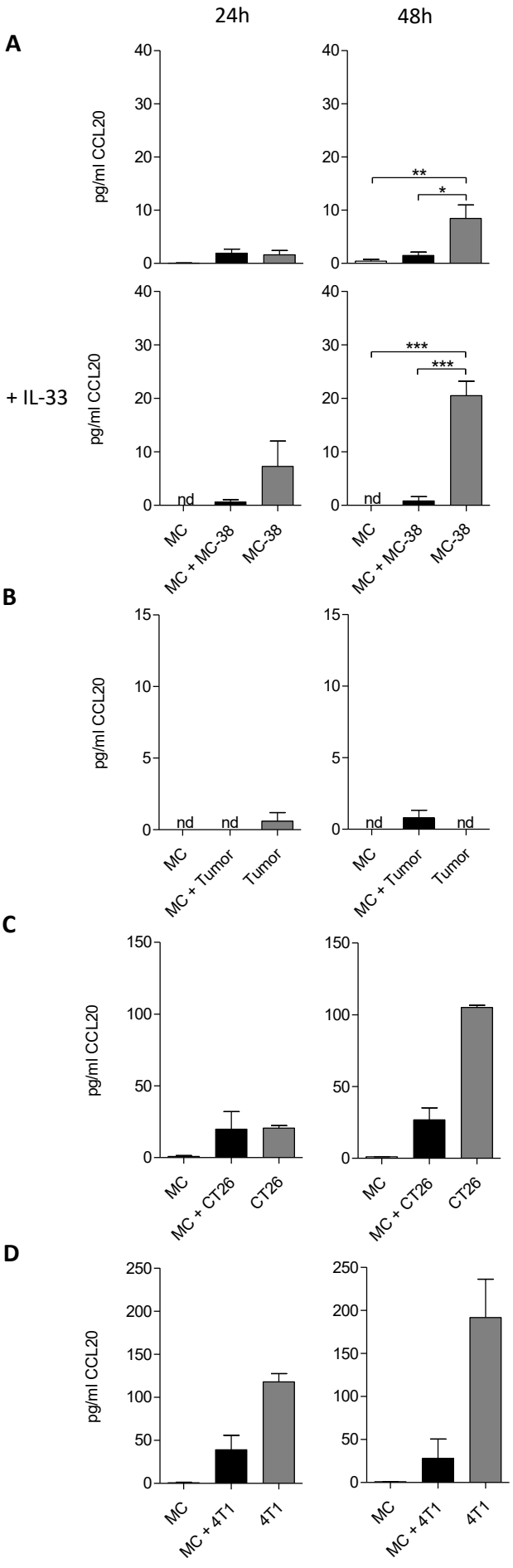


Figure 59: CCL20 chemokine released by cancer cells is consumed in the co-culture system. CCL20 was measured by ELISA in 24 and 48h supernatants derived from co-cultures between BMMCs (MC) and MC-38 cells in the presence or absence of 50 ng/ml IL-33 **(A)** or adherent cells derived from MC-38 tumor mass (Tumor) **(B),** CT26 **(C)** or 4T1 **(D)** cell lines cultured alone or in co-culture. Reported results are mean (+ SEM) from 2 (in CT26 and 4T1 analyses) or at least 3 (in MC-38 and tumor cells analyses) independent experiments. *p<0.05, *p<0.01 by one way ANOVA. nd = not detected.

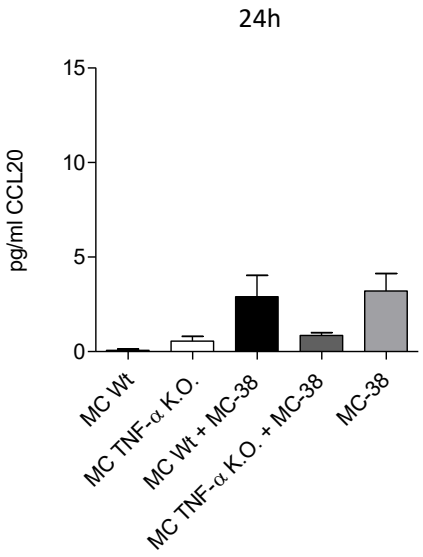


Figure 60: Higher levels of CCL20 are detected in the culture medium of MC-38 in the presence of Wt MCs compared to TNF-α K.O. MCs. CCL20 was measured by ELISA in 24h supernatants derived from MC-38 cell line cultured alone or with Wt or TNF-α K.O. BMMCs. Reported results are mean (+ SEM) from two independent experiments.

6. DISCUSSION

MCs are innate immune cells that are highly shaped by different local tissue environments. This aspect is arguably the most modern paradigm of MC's biology, as is stated multiple times in this thesis. Indeed, these cells acquire their mature phenotype in the periphery after the migration of immature MCs precursors and virtually populate all vascularized tissues of mouse and human bodies. As a consequence, MCs manifest great plasticity in phenotype and functions and high "sociality" with other cell types[5].

The main contribution of this work is providing concrete demonstrations that there is a reciprocal functional influence between MCs and microenvironmental cells, in particular, MCs and B cells are strict partners contributing to homeostatic and pathological responses, such as colitis and CRC that are studied in this thesis.

Most of the literature about MC-B cell interplay conducted *in vitro* made use of immature BMMCs and conventional B-2 lymphocytes, however it is important to consider that also B cell types, like MCs, vary according to the anatomical site. Mouse peritoneal cavity and intestine are good examples because these compartments are B-1 enriched sites and are populated by two different MCs sub-types, serosal- and mucosal-MCs respectively. This is the reason why we decided to extend the conventional BMMCs/B-2 cells cross-talk analysis with PDMCs and peritoneal cavity B cells in our co-culture system.

Analysing the most known MCs' anaphylactic response, the degranulation upon an IgE/Ag stimulation, we observed that it was slightly altered in the presence of both resting or activated B cell subsets (*figure 21*). A major effect instead was observed both on BMMCs and PDMCs' surface molecules that were modulated by B cells (*figure 22*). MHC-II, that is almost absent on resting MCs and upon an IgE/Ag-dependent activation, was upregulated after culturing MCs with both sub-populations of B cells. We can speculate that, *in vivo*, MCs acquire APCs ability after interacting with B cells. In turn, antigen sensitized T cells could efficiently stimulate B cells in GC reactions: this would finally favour B cells' effector functions and terminal differentiation. Another possible explanation is that this increase is not due to an endogenous MHC-II overexpression on MCs, but results from the transfer of MHC-II proteins in vesicles from B cells during the formation of a synapse-like structure, as it has recently been shown to happen *in vivo* between MCs and DCs in inflamed skin[315].

Another surface marker that was up-regulated on the surface of MCs was ST2, the membrane receptor for IL-33. This modulation was obtained by the sole effect of the co-culture with B-1

enriched cells. This suggests that an ST2^{high} phenotype of MCs might be favoured in body environments in which they are in contact with innate B lymphocytes such as the peritoneal cavity and the intestine. During the early phases of tissue injury, MCs are early targets of IL-33 since they are the first tissue-resident "sentinel" cell that constitutively express ST2[316]. The importance of an IL-33 dependent activation of MCs is further highlighted in the analysis of the cross-talk between MCs and MC-38 cancer cells. When we analysed the release of pro-inflammatory mediators by MCs upon stimulation by cancer cells and the mutual-induced regulation of cancer-related chemokines, we added this factor to mimic the CRC milieu (*figures 50-57*). MCs and MC-38 cells respective activations were potentiated concurrently with the addition of IL-33, however the contribution given by the interplay with the other corresponding cell type was still noticeable. It is important to remember that IL-33 is not only an important component of tumor growth (whose effect is still debated[317,318]), as a matter of fact, it is also released upon intestinal acute inflammation. In this regard, an ST2^{high} phenotype, a possible consequence of the cross-talk with innate-like B cells, would be essential for an adequate activation of MCs during colitis and at the early stages of tumorigenesis. By analysing MCs' related soluble mediators we uncovered novel specificities in the MCs/peritoneal cavity B cells interplay: IgE/Ag triggered MCs are sustained by B-1 lymphocytes in the neo-synthesis and release of the TNF-α, CCL2, IL-13, and IL-6 pro-inflammatory mediators (*figure 23-27*). We excluded the involvement of the membrane CD28 - CD80/CD86 axis in the increased production of these factors (*figure 28*). On the contrary, we showed that soluble thermolabile mediators derived from peritoneal cavity B cells were sufficient to generate the TNF-α enhanced production in IgE/Ag stimulated MCs (*figure 29*). Innate-like B cells are known to spontaneously release a wide range of mediators, most of them necessary for their self-sustaining and long-living (e.g. IL-10, IL-6...)[134]. Since we also obtained the ST2 up-regulation by the effect of peritoneal cavity B cells, we hypothesized that B-1 cells' mediators might promote an autocrine loop for IL-33 production in MCs. However, IL-33 K.O. BMMCs showed the same ST2 increase and augmented production of TNF-α (*figure 30*). In literature, no reports indicate that B cells are able to produce IL-33, but we cannot completely exclude that peritoneal cavity B cells could produce this cytokine. It is well known instead that peritoneal cavity B cells spontaneously release high levels of IL-10: a compelling candidate for this effect. Indeed, a link has already been shown between B cell-derived IL-10 and MCs functionality: in 2015 Kim and collaborators showed that CD5^{+} and IL-10 producing splenic B cells suppress IgE-mediated MCs' anaphylaxis and mediators release[197] but it was also shown that IL-10 can enhance MC's activation through a Stat3-dependent process[319].

To sum up, these *in vitro* data proved that peritoneal cavity B cells are important modulators of MCs' inflammatory activation. This led us to study MCs distribution in a B-1 lacking mouse model, the so-called *bumble* mice[130]. Among the organs analysed so far, in a scenario characterized by tissue hyperplasia and increased immune infiltrates (*figure 32*), MCs were observed to be unaffected in their distribution in the peritoneal cavity while they were increased in the lungs and in the jejunum of this newsworthy mouse model (*figure 31 and 33*). We also set up a culture of PDMCs from *bumble* mice but we did not observe any differences in the expression of the ST2 receptor compared to the Wt cultures (data not shown). These preliminary observations from the *bumble* mice require further studies with an extended panel of MCs characterization and functionality: it is possible that any variations in MCs' phenotype in the mouse model were lost after culturing the cells. It may also be worth performing a functional test such as the passive systemic anaphylaxis (PSA). Briefly, mice are first sensitized by a systemic injection of IgE and then anaphylaxis is induced by the Ag trigger. For our purposes, TNF-α released within the peritoneum after 1h of the Ag injection would be compared between control and *bumble* mice.

Studying MCs' contribution in B cells' survival, proliferation, antibody production and up-regulation of activating membrane molecules (CD86 and MHC-II), we observed a relevant role for the sole splenic population. Indeed, we observed that peritoneal cavity B cells, which are self-sustaining, did not need MCs' support to achieve their functions (*figure 30*).

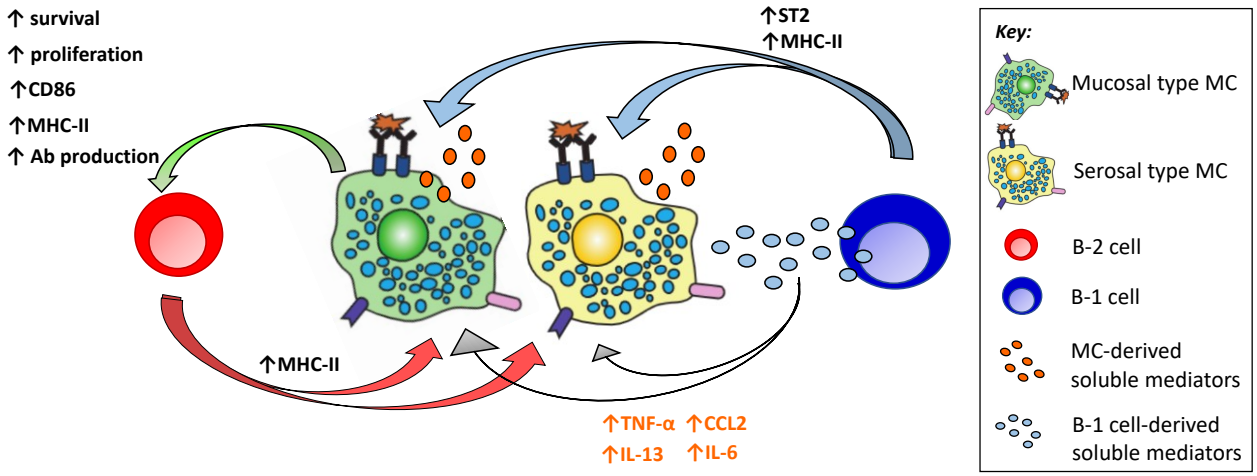


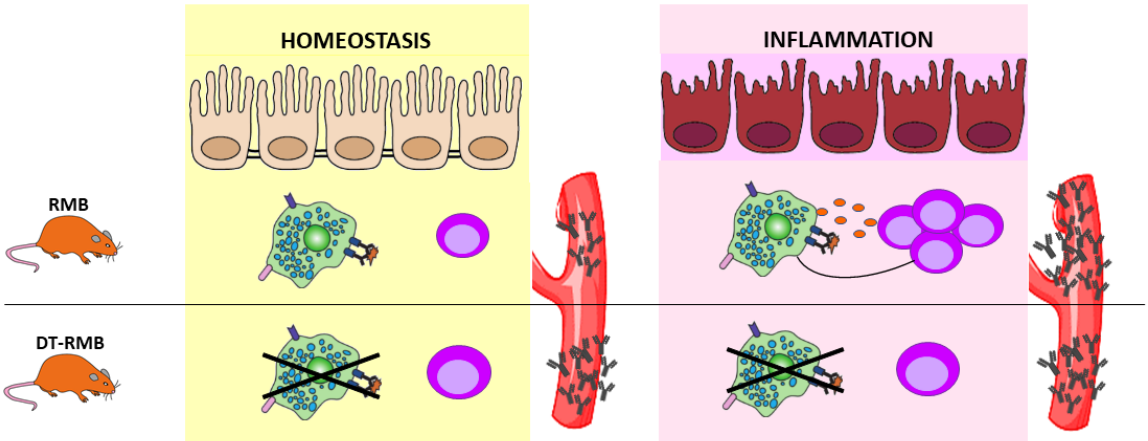
Figure 61: A novel paradigm of MC/B cell cross-talk: immature mucosal-type MCs (BMMCs) support survival, proliferation, up-regulation of the membrane molecules CD86 and MHC-II and promote the antibody production of the splenic B-2 population. On the contrary, none of these effects were reported on the peritoneal cavity B-1 enriched subset. The MHC-II expression on both mucosal and serosal-type MCs (PDMCs) is induced after their co-culture with both B-1 and B-2 lymphocytes. ST2, in addition, is overexpressed on MCs by the sole effect of B-1 cells. Moreover, soluble mediators spontaneously secreted

by innate B-1 lymphocytes are able to sustain the neo-synthesis and the release of pro-inflammatory factors such as TNF-α, CCL2, IL-13 and IL-6 from IgE/Ag activated MCs.

To analyse whether MCs have a role in the development and differentiation of B cells *in vivo*, in homeostatic conditions, we performed a systematic analysis of B cell distribution in two MCs-deficient mice models: the well-known *Kit$^{W\text{-}sh}$* mouse which lacks mature MCs from the birth of the animal, and the more recently developed MCs- and basophils-depletable RMB mouse. Our observations did not point out alterations in the distribution of B cell populations in spleen and LNs of the MC-deficient mice. No differences were observed in the absence of MCs in splenic B cells' membrane markers expression nor in their *ex vivo* ability to survive and proliferate in both models. Interestingly, differently from the RMB model, the peritoneal cavity of *Kit$^{W\text{-}sh}$* mice presented an inverted proportion of B-1 and B-2 cells (*figure 36*). This inversion was not restored upon MCs reconstitution and can be the case of an additional, but neglected, defect of this mouse model due to the germline *kit* gene mutation. However, more intriguingly, the pre-natal development of the B-1 cells could be affected by the lack of MCs. The yolk sac and the fetal liver are anatomical sites in which the B-1 lineage develops[130] and recently it was shown that the yolk sac and the liver are important sources of MCs precursors[17]. Another detected alteration in the absence of MCs was reported in Abs production. In the DT-RMB model, increased levels of IgA produced by *ex vivo* splenic B cells upon stimulation and in the serum were measured (*figure 37-38*). Moreover, IgA PCs detected by histology in the DT-RMB mice, even if comparable in numbers, were increased in dimension, probably because of an increased Abs synthesis (*figure 41*). In *Kit$^{W\text{-}sh}$* mice higher basal levels of IgA were detected in the serum and these levels were maintained upon MCs reconstitution. Altogether, these results suggest a direct or indirect role of MCs in maintaining homeostatic levels of IgA.

Next step was to set up an inflammatory model, not MCs or B cells dependent, in which both cell types are known to be activated and affected by the progression of the disease: the model of the DSS-induced intestinal acute inflammation was chosen and performed in the RMB mouse model. The intestine, as we said before, is an important environment in which MCs and B cells can cross-talk. In fact, in the past we showed in histological sections the evidence of co-localization of these two cells [196] but, to our knowledge, no *in vivo* demonstrations indicating their functional interaction in the gut are available so far. Interestingly, MCs-depleted DT-RMB mice had a worse progression of the colitis (*figure 39*). This aspect indicates a beneficial impact provided by MCs in the recovery of the acute intestinal inflammation, in agreement with what proposed in 2015 by the group of Prof.

Colombo that studied the DSS-induced colitis in *Kit*$^{W\text{-}sh}$ mice. According to Prof. Colombo's model, MCs infiltrating the colon upon DSS withdrawal upregulated ST2, in this context IL-33 activity on MCs was essential in the resolution of inflammation and MCs-derived MCPT-4 was fundamental to promote mucosal healing[233]. In our model, we focused on understanding whether MCs deficiency impacted B cells' activation in DSS-treated mice. While the central reservoir of splenic B cells' distribution and phenotype seemed not to reflect signs of activation, the levels of IgA released after the *ex vivo* culture of these cells were tendentially increased in DSS-RMB mice. A clearer evidence of IgA increase upon DSS treatments was observed systemically in the serum, and in the increased accumulation of IgA$^+$ PCs provided by the histological sections of the inflamed colons (*figure 41)*. In addition, this increase in IgA was accompanied by decreased levels of IgM in the serum, suggesting a phenomenon of isotype switching. Higher IgA production in colitis and in the DSS mouse model is, as a matter of fact, a known mechanism of anti-inflammatory response[242240]. Interestingly, this skewing towards IgA$^+$ PCs in the inflamed colon was not observed in DSS treated DT-RMB mice and neither increased in the serum, compared with their respective controls (*figure 41*). These results indicate that, in the absence of MCs, B cells were deprived of important sustaining factors for their activation. Further investigations are needed to determine the *in vivo* mechanism on which this effect is based. However, from *in vitro* data published by our group, it is known that both the co-stimulatory axis CD40/CD40L and MC-derived-IL-6 are responsible for controlling the expansion of IgA PCs[195]. It is therefore likely that the lack of MCs is accompanied by the reduction of those factors that are fundamental to sustain B cells' IgA isotype switching in a context in which these Igs would participate in the amelioration of the acute inflammatory insult at the intestinal mucosal barrier.



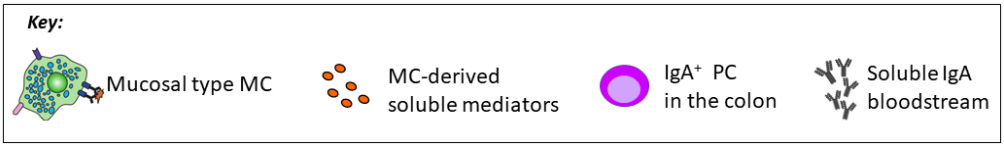


Figure 62: MCs control IgA$^+$ PCs' function in the intestine: In homeostasis, after the depletion of MCs (DT-treated RMB mice condition) the number of IgA$^+$ PCs in the colon is unaffected, however, these cells are increased in dimension. In the serum higher levels of total IgA are reported and also splenic purified B cells release spontaneously higher levels of this Ab (not shown in the figure). During inflammation, in MCs-competent mice, the number of IgA$^+$ PCs in the colon is increased and in the blood a higher level of IgA is detected compared to the homeostatic condition. When the intestinal inflammation is established in MCs-depleted mice both, the number of IgA$^+$ PCs in the colon and the amount of seric IgA are not augmented, compared to the control condition. These evidences suggest the importance of MCs in the regulation of PCs in the intestine both in healthy conditions and upon colitis. The combination of contact-mediated mechanisms and the release of soluble mediators from activated MCs could support the effector functions played by B cells in response to the damage at the intestinal barrier.

It is well known that patients suffering from IBD are exposed to increased risks to develop CRC. This cancer type, that is a major health problem and the fourth cause of cancer deaths worldwide, is accompanied by disorders of the immune system[320]. A better characterization of specific immune reactions in carcinogenesis is indeed a primary goal. MCs and B cells are important components of tumor infiltrates but whether they have tumor-promoting or anti-tumoral roles is still undefined because of conflicting results[43,252,258,262]. We aimed at determining whether MCs' phenotype and activation induced by the direct effect of cancer formation could influence B cells' behaviour.

To investigate this aspect we established a subcutaneous model of CRC in which we first characterized B cells' distribution, phenotype and activation comparing B cells from MC-38 tumor-bearing and control mice. We observed an accumulation of B lymphocytes in tumor dLNs and infiltrates of B cell in the tumor mass (*figure 42*). The accumulation of B cells in dLNs was not unexpected[305] and our group recently proposed this as a generalized mechanism related to CRC onset that joined three different set-ups of CRC. Moreover, we showed that CRC profoundly affects B cells' ongoing in tumor mice not only locally but also at the system level[263]. In this thesis, we carried out a splenic B cells' characterization analysing a set of activating and tumor-related membrane molecules. An increased expression of PDL-1 and MHC-II on tumor splenic B lymphocytes compared to control splenic B cells was observed, expression of a tumor-dependent phenotype (*figure 42*). Consistently, it has been recently shown that B cells with a high expression of PDL-1 are associated to an immunosuppressive phenotype in the CRC context[321]. We also investigated the B

cells' ability to release Abs: the basal release of IgM, IgG and IgA isotypes from tumor-bearing mice purified splenic B cells was tendentially increased.

By exploring B cells accumulation in dLNs, we hypothesized an increased chemotaxis since in our previous work we demonstrated that the enhanced proliferation rate was not the explanation of the accumulation of B cells in dLNs[263]. Therefore, we evaluated the expression of chemokine receptors on splenic B cells of tumor-bearing mice and healthy controls, but no differences were observed, suggesting that B cells have the same ability to home peripheral lymphoid structures (*figure 43*). We then assessed the expression of B cells-related chemokines in LNs and, interestingly, increased expressions of and CCL20 and CXCL13 were detected in tumor-dLNs compared to control mice LNs (*figure 43*). This suggested a role of these two factors in the accumulation of B lymphocytes in dLNs.

In the light of these results, we focused on our main goal: determining if MCs could affect the behaviour of B cells upon tumorigenesis. To this end, we repeated the same experiments by comparing the analyses of B cells from Wt, *Kit*$^{W\text{-}sh}$ and rec-*Kit*$^{W\text{-}sh}$ tumor mice. Again, B cell accumulation in dLNs compared to ndLNs was observed in all three tumor-bearing mice models. Interestingly, we noticed a tendentially reduced percentage of B cells in the spleen and dLNs of rec-*Kit*$^{W\text{-}sh}$ compared to the *Kit*$^{W\text{-}sh}$ tumor-bearing mice. This aspect may indicate a preferential B cells accumulation in other compartments, for instance in the tumor. Indeed, by flow cytometry, we observed increased B cell infiltrates in the tumor of rec-*Kit*$^{W\text{-}sh}$ mice compared to *Kit*$^{W\text{-}sh}$ and Wt mice. In addition, according to histological analyses, tumor infiltrating CD45R^{+} elements are strongly reduced in the *Kit*$^{W\text{-}sh}$ tumors compared to the Wt condition, while in in rec-*Kit*$^{W\text{-}sh}$ mice tumors a tendency to restore the Wt condition was obtained (*figure 44*). These observations indicate the presence of MCs as a needful element for the correct integration of B cells in the TME. In 2016, in line with our hypothesis, Palm and collaborators suggested that MCs have a role in enhancing the capacity of B cells homing to inflammatory sites by inducing their up-regulation of surface L-selectin[198]. Concerning our model, we are able to advance both an indirect and a direct role of MCs in promoting B cells' homing in TME.

Specifically, the increased expression of and CCL20 and CXCL13 in dLNs observed in Wt tumor mice, was not detected in *Kit*$^{W\text{-}sh}$ tumor-bearing mice; on the contrary, it was restored upon MCs reconstitution of *Kit*$^{W\text{-}sh}$ mice (*figure 46*). In support to our observations in mouse studies, by analysing a human database of CRC-derived cell lines, we found higher CCL20 expression in LNs metastasis compared to the primary tumor (see *Appendix* section). CCL20 is indeed known to

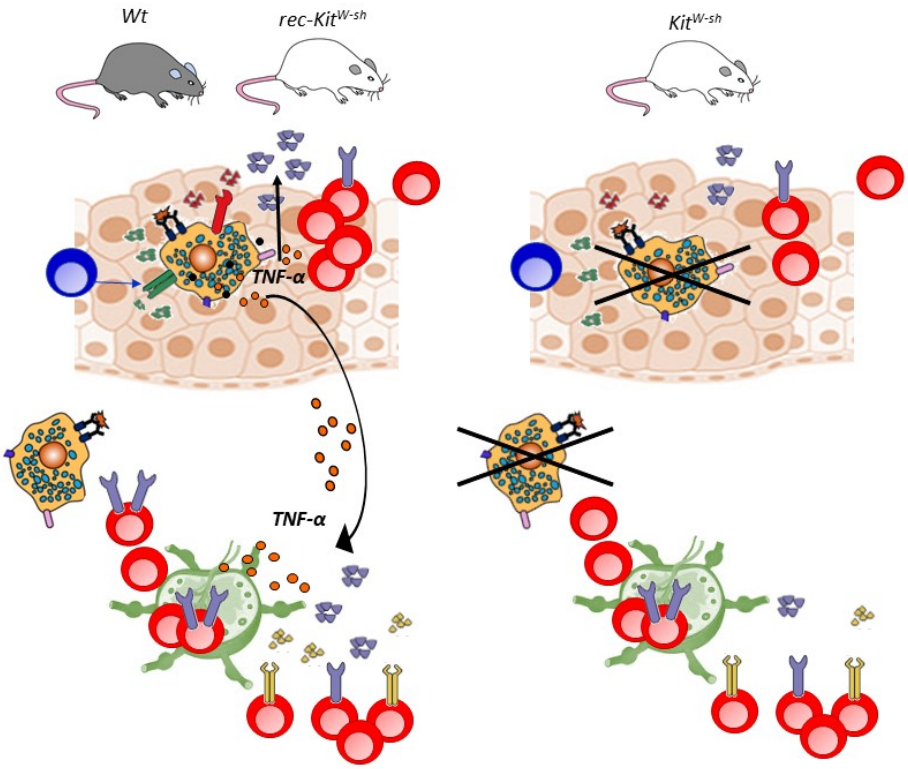
promote cancer spreading and metastasis, as it has recently been confirmed[322]. Moreover, CCR6 (CCL20 receptor) expression on B cells that accumulate in dLNs is higher if compared to ndLNs in all the three mice groups. This corroborates the thesis that CCL20 may attract B cells in the tumor dLNs and, since the expression of its receptor is even higher in rec-*Kit*$^{W\text{-}sh}$ mice, the presence of MCs is somewhat relevant (*figure 47*). It is useful to correlate this data with the analyses done on chemokine receptors modulation on B cells in the framework of the interplay with MCs: CCR6 resulted up-regulated by the effect of activated BMMCs (*figure 35*). All these results strongly suggest that increased chemokine levels promote B cells accumulation in dLNs and that MCs are involved in the generation of a B cells attracting microenvironment at the levels of tumor and dLNs. Moreover, CCL20 involvement in our model was also confirmed by analysing serum levels of the chemokine that in Wt tumor mice rise upon tumor development, while its increase is not equally reproduced in *Kit*$^{W\text{-}sh}$ tumor-bearing mice *(figure 48*).

CCL20 has been reported to attract tumor-promoting immunosuppressive cells (e.g. Treg) to the tumor microenvironment[273]. As a future perspective of this study, it would be interesting to determine whether CCL20 attracts preferentially B cells with an immunosuppressive phenotype.

To address the mechanism by which MCs could be involved in creating a favourable environment for B cell infiltration, an *in vitro* co-culture system between cancer cells and MCs was used to investigate the MCs-CRC cells cross-talk. First, we uncovered that an enriched pro-inflammatory milieu was established as a consequence of the direct interplay between cancer cells and MCs. Specifically, the inflammatory TNF-α, IL-6, and IL-13 were detected in the co-culture medium as well as the potent MCs' chemotactic and activator SCF (*figure 50-55*). More importantly, we demonstrated that resting MCs release TNF-α spontaneously after interaction with cancer cells or their conditioned medium. This was an essential step in the investigation of the chemotactic mechanism. When we analysed the MCs/MC-38 cancer cells mutual interaction in the expression of chemotactic molecules, we uncovered that tumor cells that were conditioned by the presence of MCs, increased their basal expression of CCL2, CCL5 and notable CCL20. It is also worth noting that, even though murine MCs are not able to express the CCL20 chemokine (differently from human MCs[323]), their CCL5 transcriptional activity was increased after the contact with MC-38 tumor cells. This indicates that also MCs are induced to synthesize inflammatory chemokines that can co-operate in the recruitment of immune populations at the tumor sites (*figure 57*). The data obtained with CCL20 was very interesting and made us believe in a connection between MCs' activation and the generation of a CCL20 enriched tumoral environment. Very interestingly, transcriptional

analyses and ELISAs conducted in the co-culture between MC-38 cells and TNF-α K.O. BMMCs highlighted that TNF-α release by MCs is an essential factor for the CCL20 sustained expression in cancer cells (*figures 58, 60*). Our hypothesis is that MCs, attracted in the TME, participate in the generation of a pro-inflammatory environment which can recall and activate other immune cell populations. Among these factors released upon activation, TNF-α plays a role by inducing higher expression of CCL20, that in turns attracts B lymphocytes. This factor may act not only locally in the tumor but also at the level of the dLNs favouring increased expressions of CCL20 in LNs resident cells. Moreover, at the levels of the LNs also other factors, such as the CXCL13, can participate in the creation of enriched sites of B cells. With the purpose of demonstrating the actual role of MCs and of its derived-TNFα in the CCL20 induction, MC38 tumor-bearing mice established in Wt- or TNFα K.O.- BMMCs reconstituted-$Kit^{W\text{-}sh}$ mice would be suitable. Any differences in B cells accumulation and CCL20 levels in the two types of MCs-reconstituted tumor-bearing mice will reasonably be attributed to MCs.

In conclusion, the second part of this thesis, enlightened a novel and intriguing mechanism by which MCs, shaped by the tumor, could indirectly enhance B cell activation and accumulation in tumor sites (the model is proposed in *figure 63).*



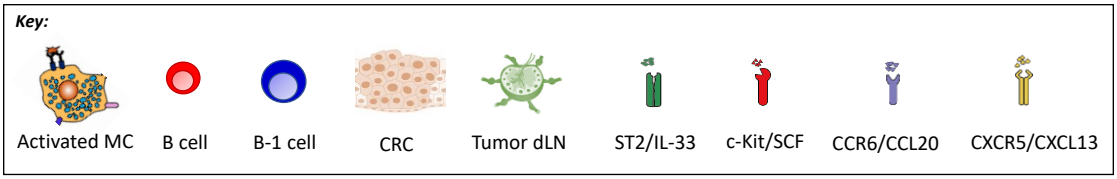


Figure 63: MCs promote the chemotaxis of B cells in CRC: On the left, CRC is established in MCs-competent mice. MCs are the first innate immune cells that as «sentinels» react to the imbalanced homeostasis. Thanks to their ability to promptly respond to tumor-derived SCF and IL-33, MCs release inflammatory factors including the TNF-α. In an hypothetical orthotopic scenario, B-1 cells that are known to home the gut, can favour a faster and stronger activation of MCs (e.g. by inducing ST2 up-regulation on the membrane of MCs). The TNF-α released in the TME sustains the production of the CCL20 chemokine that attracts B cells and other leukocytes. TNF-α released at the tumor level, may induce further production of CCL20 in dLNs. CCL20, together with CXCL13 and other chemotactic factors, recall B lymphocytes. On the right, in MCs-deficient tumor mice, because of the lack of the MC's contribution in enhancing CCL20 production in the tumor, reduced TIBs are reported. Moreover, a reduced increase of CCL20 and CXCL13 are also reported in the dLNs. B lymphocytes equally accumulate in the dLNs thanks to the contribution of other factors that are produced upon tumor formation.

An aspect to take into account when analysing immune cells in tumor setting is the phenotype that they acquire which, as said before, can reflect tumor -promoting or tumor-suppression hallmarks. B cells, depending on the phenotype and the priming by other cell types (e.g. by myeloid derived suppressor cells (MDSCs) in the context of breast cancer[324]), are known to play pro-tumoral or anti-tumoral effects. By exploring phenotypical surface markers of splenic B cells in rec-*Kit*$^{W\text{-}sh}$ tumor-bearing mice (beyond the already mentioned PD-L1) higher FasL levels were detected (*figure 45*). This molecule is included among the markers associated with a "killing" phenotype of B cells in cancer[259]. Nevertheless, the phenotype of B cells that accumulate in the tumor and dLNs is our system is still an open question and an important perspective of the study.

The take-home message from this work is on the one hand that a complex cross-talk is established between MCs and different B cell subtypes: MCs during an allergic IgE/Ag dependent activation may find a support in anatomical sites populated by innate B lymphocytes which provide exacerbating factors for the activation of MCs. On the other hand, concerning MC's activation, the presence of this "sentinel" cell is important in conditions of acute insults, such as intestinal colitis and also in CRC formation. MCs-derived factors, upon appropriate and prompt activation, are known to be essential in the tissue repair during acute intestinal insults (e.g. through the action of MCPT-4[233]) and, as we propose in addition, they are essential partners for intestinal B cells sustenance in the production of mucosal protective IgAs. Moreover, during cancer development MC's pro-

inflammatory mediators, by enhancing the expression of chemotactic factors both in the tumor and in dLNs, are also indirectly responsible for B cells accumulation in tumoral sites.

In conclusion, we hope that our study will provide new strategies to target the generally ignored "rheostatic" ability of MCs to range over activation to suppression of inflammation, which, if appropriately manipulated, could lead to new therapeutic approaches for human diseases such as IBD and CRC.

7. MATERIALS AND METHODS

7.1. SOLUTIONS AND CULTURE MEDIA

Wash medium	RPMI 1640 (Euroclone); 10% FBS (Sigma Aldrich); 20 mM Hepes (Euroclone); 2 mM L-glutamine (Euroclone); antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) (Euroclone)
Complete culture medium for B cells and co-culture experiments	RPMI 1640 (Euroclone); 10% FBS (Sigma Aldrich); 20 mM Hepes (Euroclone); 2 mM L-glutamine (Euroclone); 1 mM sodium pyruvate (Euroclone); 1X non-essential amino acids (from 100X mix, Euroclone); antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) (Euroclone); 50 mM β-mercaptoethanol (Sigma Aldrich)
Complete culture medium for BMMCs and PDMCs	RPMI 1640 (Euroclone); 20% FBS (Sigma Aldrich); 20 mM Hepes (Euroclone) 2 mM L-glutamine (Euroclone); 1 mM sodium pyruvate (Euroclone); 1X non-essential amino acids (from 100X mix, Euroclone); antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) (Euroclone); 50 mM β-mercaptoethanol (Sigma Aldrich); 20 ng/ml IL-3 (PeproTech); 20 ng/ml SCF (PeproTech)* (**only for PDMCs cultures)*
Complete culture medium for MC-38, CT26, B16-F0, 4T1 cell lines	DMEM High Glucose (Euroclone); 10% FBS (Sigma Aldrich); 2 mM L-glutamine (Euroclone); antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) (Euroclone)
Phosphate Buffered Saline (PBS) pH 7.4	137 mM NaCl (Sigma Aldrich); 27 mM KCl (Sigma Aldrich); 4.3 mM Na_2HPO_4 (Sigma Aldrich); 1.4 mM KH_2PO_4 (Sigma Aldrich)
MACS buffer	PBS pH 7.4; 2 mM EDTA (Sigma Aldrich); 0.5% BSA (Sigma Aldrich)
Tyrode's buffer pH 7.4	10 mM HEPES buffer; 130 mM NaCl; 5 mM KCl; 1.4 mM CaCl2; 1mM MgCl2 5.6 mM glucose; 0.1% BSA

7.2 ANIMALS, CELL PREPARATION AND CULTURE CONDITIONS

7.2.1 Mice and treatments

C57BL/6 and BALB/c mice were purchased from Envigo (Netherlands) and maintained at the animal facility of the Department of Medical Area, University of Udine (Italy). TNF-$\alpha^{-/-}$, IL-$6^{-/-}$, IL-$33^{-/-}$ femurs and tibiae were kindly gifted by Dr. Armaka M. (BSRC "Alexander Fleming", Vari, Greece), Prof. Colombo M. (Fondazione IRCCS "Istituto Nazionale dei Tumori", Milan, Italy), Prof. Bulfone-Paus S. (University of Manchester, England), respectively. Experiments with BMMCs derived from CCL2-eGFP transgenic mice and the study of B cells in *Kit*$^{W\text{-}sh}$ and RMB mice in physiologic conditions and DSS-induced intestinal inflammation were performed in the laboratory of Prof. Blank U. (University Paris Diderot, faculty of Medicine, CRI, Paris, France) during the exchange period of my PhD. *Kit*$^{W\text{-}sh}$ and BMMCs-reconstituted *Kit*$^{W\text{-}sh}$ (rec-*Kit*$^{W\text{-}sh}$) MC-38 tumor-bearing mice were done in

collaboration with Dr. Jachetti E. (Fondazione IRCCS "Istituto Nazionale dei Tumori", Milan, Italy). Organs from IκBNS-deficient *bumble* mice and their wt counterparts were kindly provided by Prof. Karlsson Hedestam G. (Karolinska Institutet, Stockholm, Sweden).

Mice were maintained under pathogen-free conditions and all animal experiments were performed in accordance to institutional guidelines and national laws of respective institutes.

8 weeks-old RMB mice were injected i.p. twice (2 days apart) with 1 μg DT (Merch Millipore) in PBS (control RMB mice were injected with PBS), and sacrificed 3 weeks after MC depletion (basophils were recovered at day 12 and mice were left one more week in conditions deprived of MCs before the sacrifice). Blood, spleen, peritoneum, LNs and colon were recollected and analysed. RMB mice were used for DSS-induced colitis experiments. 14 days after DT injections mice were administered 2% DSS (molecular weight 36,000–50,000; MP BIOMEDICALS) in drinking water for 8 days. Body weight was daily monitored to follow disease course and clinical signs of disease (consistency of the stools and rectal bleeding) were checked. At day 8 mice were sacrificed and organs were collected.

To induce tumors, MC-38 cells (2×10^5) resuspended in sterile PBS were injected i.d. in C57BL/6 wt, *Kit*$^{W\text{-}sh}$ or rec-*Kit*$^{W\text{-}sh}$ mice flanks. Control mice were injected with sterile PBS. Within 4-5 weeks of inoculation, when tumors reached the size of 1x2 cm, mice were sacrificed and blood, spleen, peritoneum, LNs and tumors were collected for *ex vivo* analysis.

7.2.2 Bone marrow-derived MCs differentiation, peritoneal-derived MCs expansion and reconstitution of *Kit*$^{W\text{-}sh}$ mice

BMMCs were obtained from 5- to 8-week-old female mice by *in vitro* differentiation of bone marrow derived progenitors obtained from mice femurs and tibiae. Precursor cells were cultured in complete culture medium for BMMCs at 37°C in 5% CO2 atmosphere. After 5 weeks, BMMCs differentiation was confirmed by flow cytometry.

Peritoneal derived mast cells (PDMCs) were isolated from 5- to 8-week-old female mice and expanded *in vitro*. Ice cold PBS, supplemented with 3% fetal bovine serum (FBS) and antibiotics, was injected into mouse peritoneal cavity, the peritoneum was gently massaged to dislodge any attached cells into the PBS solution and fluid was collected. Cells suspension was centrifuged twice (300g for 10 min at 4°C) then pellet of cells was resuspended in complete culture medium for PDMCs. At day 3, non adherent cells were discarded and fresh medium was added to the flask; at day 10 suspension cells were analysed for purity by flow cytometry. Anti- FcεRIa and c-Kit

conjugated antibodies were used to assess MCs purity. BMMCs and PDMCs cultures were usually around 96-98 % c-Kit$^+$ and FcεRIa$^+$ cells as shown in *figure 64*.

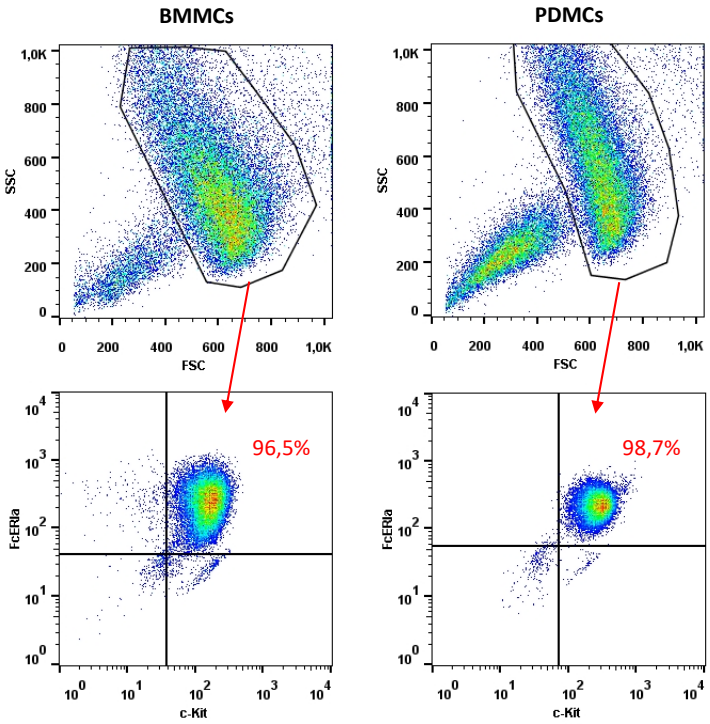


Figure 64: Purity of BMMCs and PDMCs. BMMCs and PDMCs are selected on FSC vs SSC plot and their positivity for FcεRIa and c-Kit is shown in the dot plots below.

Kit$^{W\text{-}sh}$ mice were reconstituted with i.p. injection of 5x10^6 mature BMMCs resuspended in PBS 4 weeks before their sacrifice (at the age of 11-12 weeks) for B cells analyses or the injection of MC-38 cells (see paragraph 6.2.1).

7.2.3. MCs degranulation assay

Degranulation response was determined as the percentage of β-hexosaminidase released and used as a functional test for IgE/Ag-dependent MCs activation. 0.25x10^6 BMMCs were sensitized in complete RPMI medium for 3 hours with 1 µg/ml of dinitrophenol (DNP)-specific IgE, then washed twice, resuspended in Tyrode's buffer with or without an equal amount of B cells and then challenged with 50, 10 or 1 ng/ml DNP (Sigma-Aldrich). The enzymatic activity of the released β-hexosaminidase was assessed by the cleavage of its synthetic substrate (*p-nitrophenyl N-acetyl-glucosamide*, Sigma-Aldrich) in *p-nitrophenol,* measuring the *p-nitrophenol* absorbance at 405nm

with a plate spectrophotometer. Results are expressed as the percentage of β-hexosaminidase released in the supernatant over β-hexosaminidase retained in the cytoplasm.
Leukotrienes C_4, D_4 and E_4 were measured in the same samples using a commercial detection kit (GE Healthcare) according to manufacturer's instructions.

7.2.4. B cells purification and culture

Purified splenic and peritoneal cavity B cells were obtained from 8-to-12 week-old mice. Spleens were removed and mechanically dissociated in PBS supplemented with 0.0192 M sodium citrate through 70 µm-pore-size nylon filters. Splenocyte suspension was depleted of red blood cells (RBCs) by hypotonic lysis with ACK lysing buffer (Lonza). Peritoneal cells were collected by injecting ice cold PBS, supplemented with 3% FBS, into mouse peritoneal cavity. The peritoneum was gently massaged and fluid was collected. Cells pelleted and resuspended in MACS buffer and two different strategies of B cell purification were used, both based on magnetic-activated cell sorting.
In the experiments in which B-2 and B-1 enriched B cell populations were co-cultured with MCs, a kit based on positive selection for CD19 marker, the *CD19 MicroBeads mouse* (Miltenyi, 130-052-201) was used following manufacturer's instructions. Purified splenic and peritoneal cavity B cells were cultured at the final concentration of $1x10^6$ cell/mL in complete RPMI medium.
Splenic B cells from *Kit$^{W\text{-}sh}$*, RMB and from MC38-tumor bearing mice were purified by negative selection through the use of the *B cell isolation kit* (Miltenyi, 130-090-862) following manufacturer's instructions. Purified splenic B cells were cultured at the final concentration of $1x10^6$ cell/mL, in the presence or absence of 1 µg/mL anti-mouse CD40 mAb (BD Pharmigen), 10 µg/mL LPS (Sigma-Aldrich) or 5 µg/mL CpG (Sigma-Aldrich). B cells are about 95-99% pure.

7.2.5. Cell lines

MC-38 colon adenocarcinoma cell line (C57BL/6 background) was kindly gifted by Prof. Bronte V. (University of Verona, Italy), these cells were used for *in vitro* and *in vivo* tumor experiments. C57BL/6 skin melanoma B16-F0 (ATCC^R CRL-6475^{TM}), BALB/c colon cancer CT26 (ATCC^R CRL-2638^{TM}), mouse breast tumor 4T1 (ATCC^R CRL-2539^{TM}) were used for *in vitro* experiments. All the cell lines were cultured in DMEM and Trypsin-EDTA 1x (Euroclone) was used for cell dissociation.

7.2.6. Isolation of LNs cells

Single cell suspensions from LNs were obtained by mechanical dissociation of the organs in cold PBS supplemented of antibiotics through 70 µm-pore-size nylon filters.

7.2.7. Isolation of tumor cells

MC-38 tumors were collected and suspension cells were obtained by digesting the mass with 0.25 mg/ml Collagenase type IV (Sigma-Aldrich) and 5 U/ml DNase (Roche) in RPMI supplemented with L-glutamine and antibiotics at 37°C and 5% CO_2. FBS was added to stop the reaction after 30-45min. Cells, filtered through 70 µm-pore-size nylon filters, were resuspended in fresh complete RPMI medium and used for flow cytometry analysis or plated into cell culture multi-well plates in order to have 25% confluence for co-culture experiments.

7.2.8. Co-cultures

For all co-culture experiments with B cells, MCs were previously starved for 3h in complete RPMI medium in resting condition or sensitized with DNP-specific IgE. After 3h cells were washed twice and resuspended in fresh medium alone (for resting MCs) or with 100ng/ml DNP to challenge the cells. MCs were then put in culture in a 1:1 ratio with freshly purified B cells in cell culture multi-well plates (SARSTEDT), each cell type in a concentration of $1x10^6$/ml. B cell conditions alone were included among the experimental conditions. Where indicated 2,5 µg/ml E18 Fab anti-CD28 Ab was used in the co-culture medium.

When co-cultured with cancer cells, MCs were previously starved for 1h in complete medium, resuspended in fresh medium at $1x10^6$/ml concentration and then added to the cell culture multi-well plates. All the cancer-cell used, namely MC-38, adherent cells derived from MC-38 tumors, CT26, B16 or 4T1, were plated the day before at 25% of confluence. In some conditions 50 ng/ml IL-33 (ImmunoTools) was added to the culture medium.

Cells and supernatant were collected and analysed at different time points.

7.3. FLOW CYTOMETRIC ANALYSIS AND GATING STRATEGIES

To assess cell-surface expression of different phenotypic and activation markers or co-stimulatory molecules, cultured cells were collected into polystyrene tubes (Sarstedt), washed and resuspended with PBS. After the addition of fluorescent mAbs, or Ig isotype-matched controls, cells were incubated in darkness for 30 minutes at 4°C. After the incubation, cells were washed with PBS and flow cytometry analysis were performed. FACSCalibur or FACSCanto flow cytometers (Becton Dickinson) were used for sample acquisitions. A complete list of the antibodies used in this work are shown in *table 6*.

Table 6. Antibodies used for immunophenotyping and gating strategies

Specificity of Antibody	Isotype	Clone	Conjugation	Manufacturer
CCR10	Rat IgG2b	248918	PE	R&D systems
CCR4 (CD194)	Armenian Hamster IgG	2G12	PE	BioLegend
CCR6 (CD196)	Armenian Hamster IgG	29-2L17	PE	BioLegend
CCR7 (CD197)	Rat IgG2a	4B12	PE	eBioscience
CCR9 (CD199)	Mouse IgG2a	CW-1.2	PE	BioLegend
CD117 (c-Kit)	Rat IgG2b	ACK2	FITC, PE-Cy7, APC	BioLegend
CD138	Rat IgG2a	281-2	PE	BD Pharmigen
CD19	Rat IgG2a	6D5	FITC, APC	BioLegend
CD1d	Rat IgG2b	1B1	PE	eBioscience
CD21	Rat IgG2b	7G6	APC	BD Pharmigen
CD23	Rat IgG2a	B3B4	PE	BD Pharmigen
CD28	Golden Syrian Hamster IgG	37.51	APC	eBioscience
CD30L	Rat IgG2b	RM153	PE	eBioscience
CD4	Rat IgG2a	RM4-5	FITC	BD Pharmigen
CD40L (CD154)	Hamster IgG	MR-1	PE	BioLegend
CD45R (B220)	Rat IgG2a	RA3-6B2	APC, PE/Cy7	BioLegend
CD45R (B220)	Rat IgG2a	RA3-6B2	PE/Cy7	BD Pharmigen
CD5	Rat IgG2a	53-7.3	FITC	BioLegend
CD80	A. Hamster IgG	16-10A1	APC	BioLegend
CD86	Rat IgG2b	PO3	APC	BioLegend
CXCR4 (CD184)	Rat IgG2b	L276F12	APC	BioLegend
CXCR5 (CD185)	Rat IgG2a	SPRCL5	PE	eBioscience
FasL	Hamster IgG	MFL3	APC	eBioscience
FcεRIa	Hamster IgG	mar-01	PE	BioLegend
ICAM-I	Rat IgG2b	YN/1.7.4	FITC	eBioscience
IgA	Rat IgG1	C10-3	FITC	BD Pharmigen
IL-6	Rat IgG1	MP5-20F3	PE	eBioscience
LAMP-1 (CD107a)	Rat IgG2a	1D4B	APC	BioLegend
MHC-II	Rat IgG2b	M5/114.15.2	PE	BioLegend

OX40L	Rat IgG2b	RM134L	PE	BioLegend
PD-1	Rat IgG2a	29F.1A12	PE	BioLegend
PD-L1	Rat IgG2a	10F.9G2	PE	eBioscience
ST2	Rat IgG2a	RMST2-2	APC	eBioscience
TLR2	Rat IgG2b	6C2	PE	eBioscience
TLR4	Rat IgG2a	MTS510	PE-Cy7	BioLegend
TNF-α	Rat IgG1	MP6-XT22	PE	BD Pharmingen
Isotypes controls		**Clone**	**Conjugation**	**Manufacturer**
Rat IgG1		11-430182	FITC	eBioscience
A. Hamster IgG		400907	PE	BioLegend
Mouse IgG1		11-4714-81	FITC	eBiosciences
Rat IgG1		17-4301	APC	eBioscience
Rat IgG2a		400505	FITC	BioLegend
Rat IgG2a		400508	PE	BioLegend
Rat IgG2a		400509	PE-Cy5	BioLegend
Rat IgG2b		400633	FITC	BioLegend
Rat IgG2b		12 4031	PE	eBioscences
Rat IgG2b		400611	APC	BioLegend
S. A. Hamster IgG		17-49-1481	APC	eBiosciences

7.3.1. Co-cultures experiments

Double staining strategies were used to discriminate the two different cell types in MCs-B cells co-cultures: anti- c-Kit or -CD19 mAbs were used to distinguish MCs and B cells respectively, as shown in *figure 65*. In MCs-cancer cells co-cultures, suspension cells were collected and analyses were performed on c-Kit$^+$ cells.

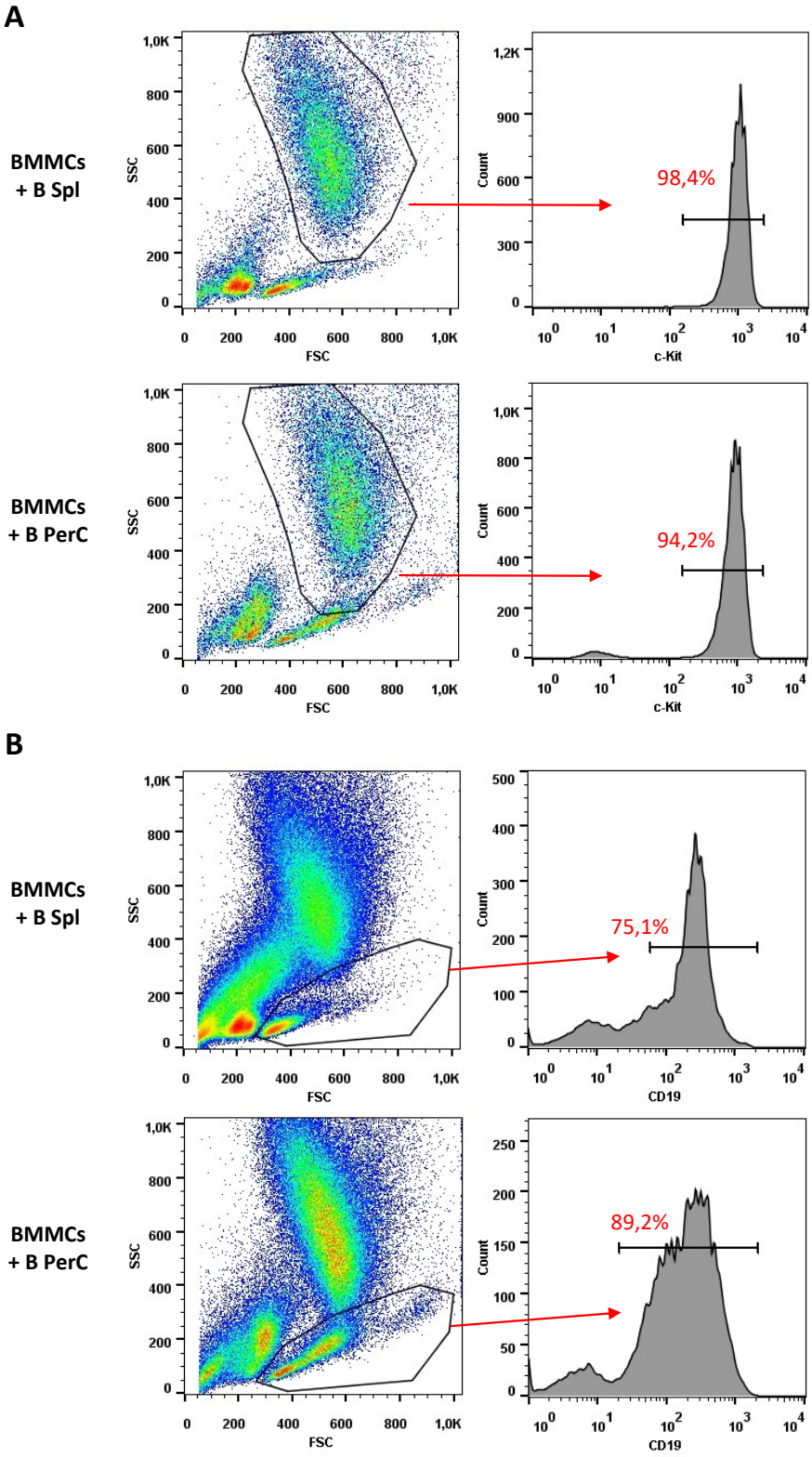


Figure 65: Examples of gating strategies used for flow cytometry analysis in MCs-B cells co-cultures. (A) BMMCs were first gated in the FSC vs SSC plot. Cells are further selected for c-Kit positivity (right panels) and on these gated cells, second membrane markers were analysed. **(B)** B cells were gated in the FSC vs SSC plot and analyses were performed on gated $CD19^{+}$ cells.

7.3.2. Splenic B cells subsets

Murine splenic B cells are heterogeneous and different subsets can be identified by the differential expression of membrane markers. The anti- CD19, CD21 and CD23 mAbs staining was used for the identification of three main naïve splenic B cells subsets: the newly formed B cells (NF), the follicular (FO) and the marginal zone (MZ) B cells[325]. In addition, transitional 2-marginal zone precursor (T2-MZP) B cell, that are $CD21^{hi}CD23^{hi}$, were identified.[326]. Furthermore MZ B cells are also characterized by the high expression of CD1d molecule. The triple staining anti- CD19, CD5 and CD1d mAbs was used to better identify other splenic phenotypes such as $CD1d^{+}CD5^{+}$ B10 cells[327]. All these subsets were analysed in MC-lacking mice to investigate any possible variations in mice carrying MCs-deficiency.

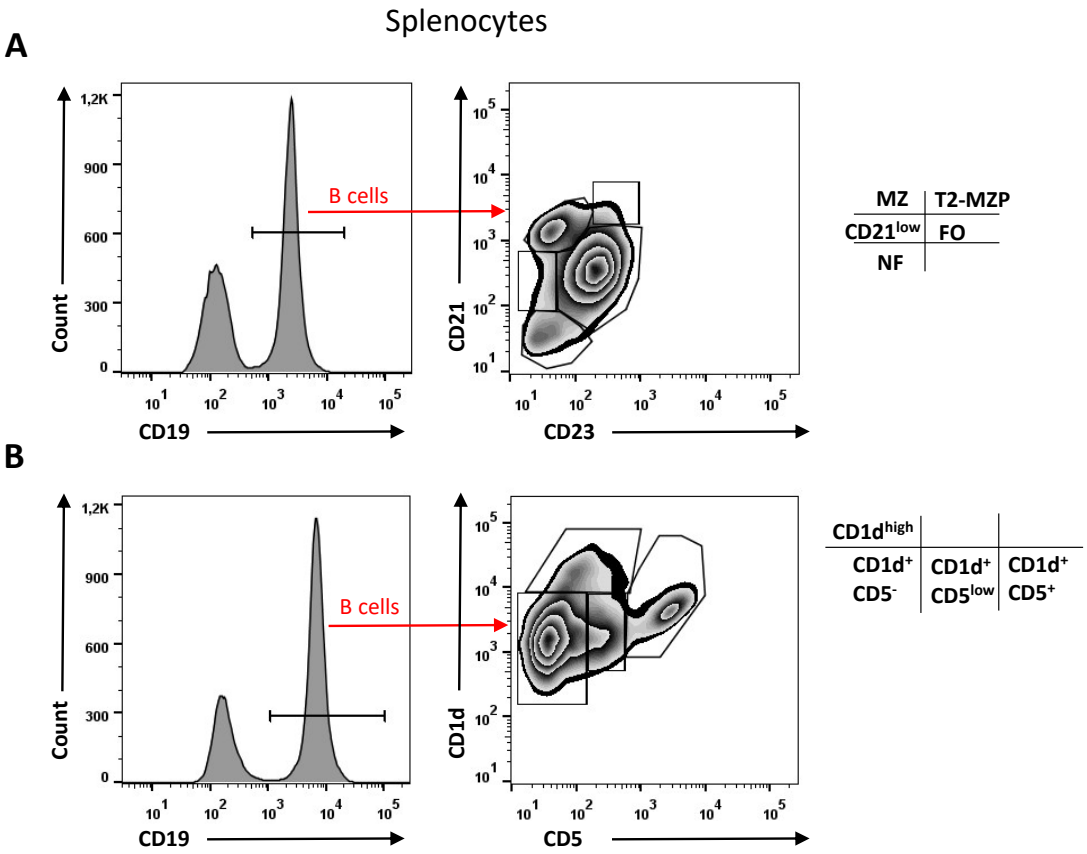


Figure 66: Examples of gating strategies used for splenic B cell subsets: B cells are first gated as $CD19^{+}$ cells among total splenocytes. Splenic B cells are further gated based on the anti- CD21 and CD23 staining in the following phenotypes: newly formed (NF), $CD21^{low}$, transitional 2-marginal zone precursor (T2-MZP), marginal zone (MZ) and follicular (FO) cells **(A)**; the anti- CD5 and CD1d staining was used to sub-divide B cells into $CD1d^{hi}$, $CD1d^{+}CD5^{-}$, $CD1d^{+}CCD5^{low}$ and $CD1d^{+}CD5^{+}$ cells **(B)**.

7.3.3. Peritoneal cavity B cell subsets

Peritoneal cavity B cells can be classified into several phenotypical and functional B cell subsets. The $CD19^+$ population contains the minor fraction of B-2 cells that is distinguished from the B-1 subset by the expression of CD23. On the contrary, the expression of CD5 by B-1a cells helps to discriminate them from B-1b cells [328]. The gating strategy used in our analyses is shown in the *figure 67*.

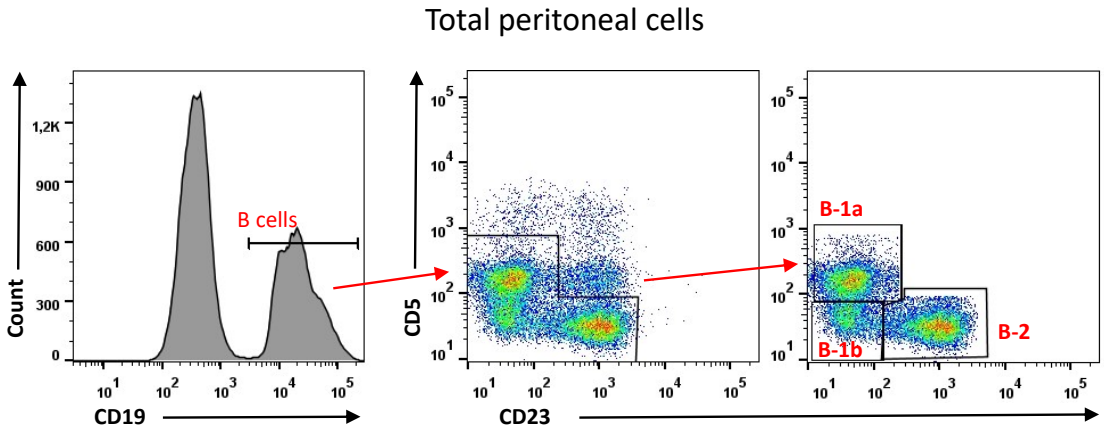


Figure 67: Examples of gating strategies used for peritoneal B cell subsets: B cells are first gated as $CD19^+$ cells among total peritoneal cells. B cells are further selected based on the anti- CD5 and CD23 staining. Among the $CD23^-$ and $CD23^+CD5^-$ cells B-1a, B-1b and B-2cells are identified as shown in the panel on the right (the analyses reported in result sections are percentages of B-1a, B-1b and B-2 cells and the total of the three is 100%).

7.3.4. B cell viability

Annexin V and Propidium Iodide (PI) staining approach (Annexin V-FITC Apoptosis Detection kit, eBiosciences) was used to determine the percentages of viable purified splenic or peritoneal cavity B cells at 48h of cultures alone in resting condition, in co-culture with MCs or upon anti-CD40, LPS or CpG stimulation. Annexin V marks cells undergoing apoptosis, dead cells instead are permeable and highly positive for PI, since they lose membrane integrity. An example of the gating strategy used to assess the B cells' percentage of viability in co-culture experiments with MCs is shown in *figure 68*.

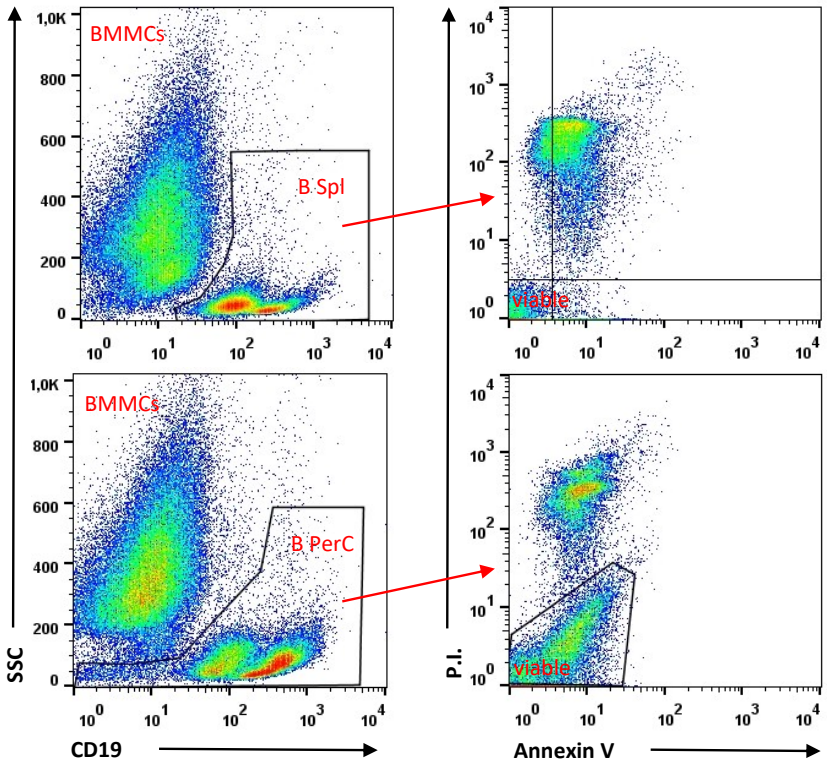


Figure 68: Strategies used for assess B cell viability in co-culture with MCs: 48h cultures between BMMCs and splenic (upper panels) or peritoneal cavity (lower panels) B cells were established. Cells were stained for anti-CD19 Ab followed by Annexin V/P.I. staining. B cells were first discriminated from MCs by the SSC vs CD19 density plot. In selected cells, viable population is determined for double negativity of both P.I. and Annexin V staining.

7.3.5. B cell proliferation

In order to study B cell proliferation, distinct generations of proliferating cells were determined by dye dilution. To evaluate the contribution of BMMCs in promoting B cells' proliferation, freshly purified splenic or peritoneal cavity B cells were stained with carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes, Invitrogen) following manufacturer's instruction, then were resuspended in fresh complete medium and cultured alone or with MCs for 72h. To assess the proliferation of *Kit*$^{W\text{-}sh}$ and RMB mice, freshly purified B lymphocytes were stained with CellTrace Violet Cell Proliferation Kit (ThermoFisher) following manufacturer's instruction and cell proliferation was analyzed by flow cytometry at 72h. The anti-CD19 conjugated Ab was used as additional B cell marker. Gating strategies performed and representative flow cytometry analyses are shown in *figure 69*.

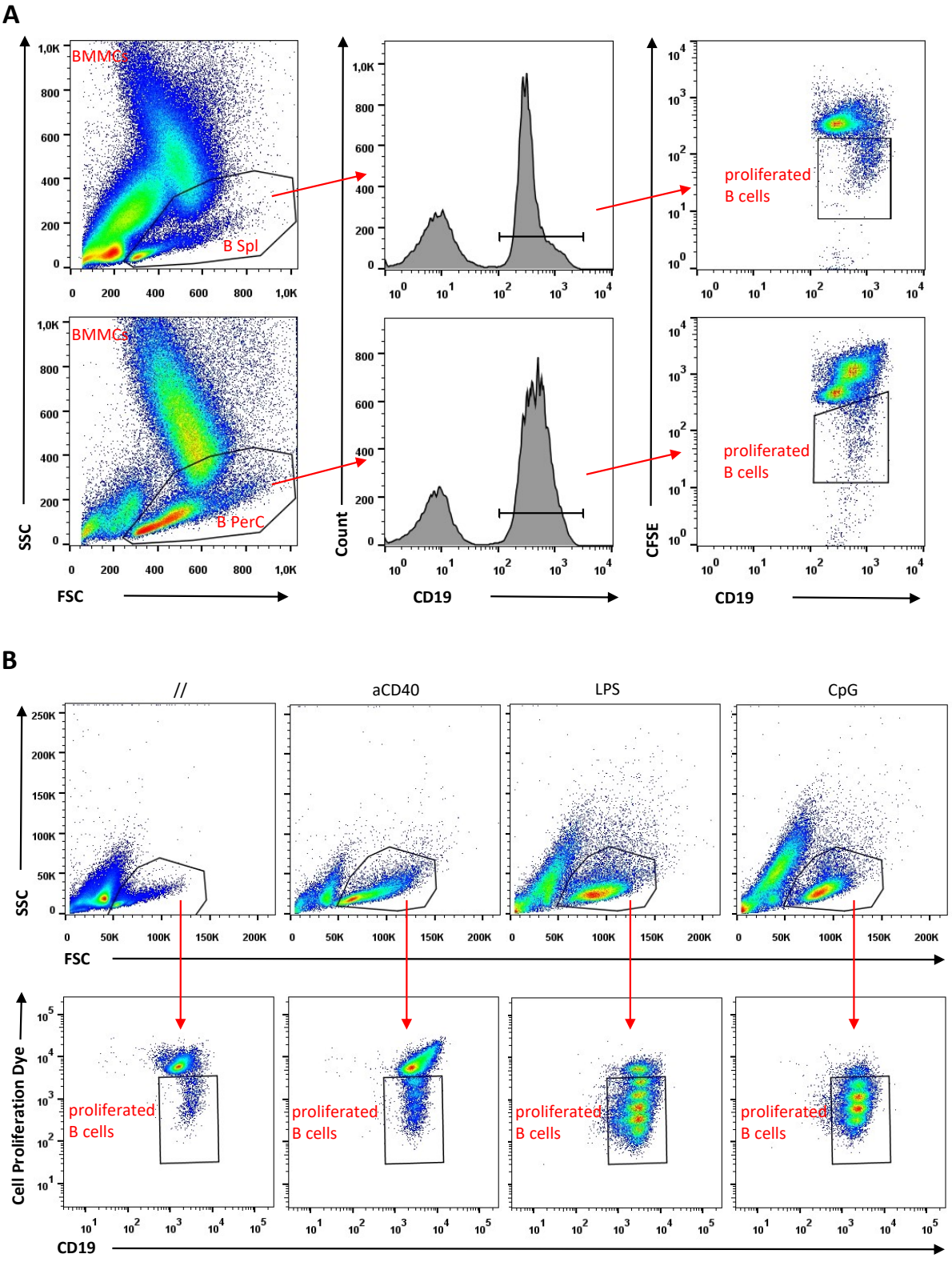


Figure 69: Strategies used to assess B cell proliferation: (A) after 72h of co-culture between CFSE-stained splenic (upper panels) or peritoneal cavity (lower panels) B cells and BMMCs, cells were stained for anti-CD19 mAb. B cells were first discriminated from MCs in the FCS vs SSC scatter plot and for CD19 positivity. Proliferated B cell population is determined by CFSE dilution. **(B)** Purified splenic B cells from a RMB mouse were stained with CellTrace Violet Cell Proliferation dye and cultured in resting condition (//) or stimulated with aCD40 mAb, LPS or CpG for 72h. Cells were then stained for anti-CD19 mAb and proliferated cells were identified in the $CD19^+$ population by Dye dilution.

7.3.6. Intracellular staining (ICS)

$0.25x10^6$ resting or IgE/Ag stimulated BMMCs are stained for intracellular TNF-α after 1 hour of co-culture with splenic and peritoneal cavity B cells in in the presence of Brefeldin A (eBioscience, 1000x solution). In order to discriminate viable from dead cells, the suspensions were stained with CellTrace™ Violet Cell Proliferation Kit (ThermoFisher SCIENTIFIC). They were then washed with PBS and incubated for 30 minutes with c-Kit mAb. Then cells were washed and fixed with 250 µl of Cytofix/Cytoperm cell fixation buffer (BD Biosciences) for 20 minutes and, at the end of the incubation, cells were washed two times with the Perm/Wash buffer for cell permeabilization (BD Biosciences). Sequentially, Fc Block antibody was added to avoid non-antigen-specific binding. After being washed, the cells were stained with Perm/Wash buffer containing anti-TNF-α mAb or Isotype control Ab and incubated for 30 minutes.

Finally, cells were washed twice with Perm/Wash buffer and analysed by flow cytometry.

For intracellular IL-6 detection in MC-38 cells cultured alone or in the presence of BMMCs, after 1h of co-culture, Brefeldin A was added overnight to the culture medium that intracellular staining was performed. In these analysis, Live/Dead Fixable Green Dead Cell Stain Probe (Molecular Probes, Invitrogen, 1:750) were used to discriminate viable from necrotic cells. A similar procedure has been followed with the addition of anti- c-kit and IL-6 mAb respectively as extra-cellular and intra-cellular markers.

7.4. QUANTIFICATION OF SECRETED MEDIATORS

Sera samples or cell supernatants for cytokine and antibody quantification were analysed by sandwich-based ELISA techniques for the detection of soluble mediators.

We used mouse IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13, IL-33 TNF-α, CXCL13 and IgM (eBiosciences), MIP-3 alpha (CCL20) and SCF (RayBiotech), CCL2 (PeproTech) specific ELISA kits according to manufacturer's instructions.

For IgG and IgA a home-made sandwich ELISA was developed. 96-well flat-bottom polystyrene plates (Corning) were coated with affinity-purified anti-mouse IgA (SouthernBiotech) or anti-mouse IgG (Sigma-Aldrich) Abs at the final concentration of 2 mg/mL and 10 mg/mL, respectively. After 1 h incubation at 37°C, plates were washed with 0.05% Tween-20 in PBS and blocked with 1% bovine serum albumin in PBS for 1 h at RT. 100 mL of cell supernatants or of opportunely diluted mouse

sera were added to Ab-coated wells. Purified mouse IgA (BD PharMingen) or IgG (Sigma-Aldrich) were used as standards. After o/n incubation at 4°C, the plates were washed and optimal concentration of horseradish peroxidase-conjugated goat anti-mouse IgA (SouthernBiotech; 1:2000) or goat anti-mouse IgG (Pierce; 1:1000) Abs were added. Next, the plates were incubated for 1 h at RT and washed before the addition of tetramethylbenzidine (TMB) substrate solution (Sigma-Aldrich). The reaction was stopped with 2 mol/L sulfuric acid and absorbance was measured at 450 nm.

7.5. RNA EXTRACTION AND RT-QPCR ANALYSES

Cells were lysed with EUROGOLD TriFast (Euroclone) and total RNA was extracted with the phenol-chloroform protocol according to manufacturer's instructions. Total RNA was quantified using a NanoDrop™ spectrophotometer (ThermoFischer) and 1µg was reverse transcribed with the SensiFAST™ cDNA Synthesis Kit (Bioline). RT-qPCR analyses were performed with SYBR Green chemistry (iQ™ SYBR Green Super Mix, BioRad) using a BioRad CFX96 real-time PCR detection systems. Target genes expression was quantified using G3PDH (glyceraldehyde 3-phosphate dehydrogenase) as normalizer gene. Primers (Sigma Aldrich) used for RT-qPCR are listed in the *table 7*.

Table 7: Primers used in real-time PCR

Target gene	Forward primer	Reverse primer
g3pdh	TCAACAGCAACTCCCACTCTTCCA	ACCCTGTTGCTGTAGCCGTATTCA
ccl2	AGCAGGTGCCCCAAAGAAGCTGTA	AAAGGTGCTGAAGACCTTAGGGCA
ccl5	TGCTGCCCACGTCAAGGAGTATTT	TCTTCTCTGGGTTGGCACACACTT
cxcl12	GGTAGCTCAGGCTGACTGGT	TCCTCTTGCTGTCCAGCTCT
cxcl13	CATAGATCGGATTCAAGTTACGCC	TCTTGGTCCAGATCACAACTTCA
ccl19	CTGTGGCCTGCCTCAGATTA	GTGTGGTGAACACAACAGCA
ccl20	TGCTATCATCTTTCACACGAAGAA	TCATTTCCTCCTTGGGCTGT
ccl21	ATCCCGGCAATCCTGTTCTC	TTCTCTTGCAGCCCTTGGAG
ccl25	CCGGCATGCTAGGAATTATCA	GGCACTCCTCACGCTTGTACT
ccl28	AGACACCTCAGTGCAACAGC	TCACCTGAGTCATTGCCAGA
il-33	CTACTGCATGAGACTCCGTTCTG	AGAATCCCGTGGATAGGCAGAG
aid	AAGTCACGCTGGAGACCGAT	AGGTAGGTCTCATGCCGTCC

7.6. IMMUNOHISTOCHEMISTRY ANALYSIS

Histologic analyses were carried out on paraffin-embedded tissues. For immunohistochemical analyses four-micrometers-thick tissue sections were deparaffinized and rehydrated.

Murine samples from Wt and *bumble* mice were stained with Hematoxylin & Eosin or with Toluidine Blue stain to determine MC distribution and frequency; MCs were counted in five nonoverlapping high-power microscopic fields (×400) and results were expressed as average.

Murine colon samples were incubated with the following primary antibody at room temperature: Goat Anti-Mouse IgA alpha chain (HRP-Conjugated), (diluition 1/400, pH9, ab97235 abcam). The binding of the primary antibody was revealed by AEC (Dako 3-amino-9-ethylcarbazole) substrate-chromogen, following manufacturer's instruction.

Double marker immunohistochemistry was performed by two consecutive rounds of single-marker. The murine tumor samples were incubated, by sequential immunostaining, with primary antibodies against Rat anti-mouse CD45R (RA3-6B2 1/10 pH9, BD Pharmigen) revealed by goat anti-rat IgG (H+L) specific secondary antibody (diluition 1/500 Novex by Life Technologies and DAB 3,3'-Diaminobenzidine Leica Biosystems), substrate-chromogen. After Fc blocking the slides were incubated with the second primary antibody Mast Cell Tryptase (EPR 8476, diluition 1:500, pH6, ab134932). The binding of the second primary antibody was revealed by Multilink Alk. Phos. Detection System (Biogenex) and Vulcan Fast Red (Biocare Medical) substrate-chromogen kit, following manufacturer's instruction. The slides were counterstained with Harris Hematoxylin (Novocastra).

All the sections were analysed under an AXIO Scope A1 optical microscope (ZEISS) and microphotographs were collected using an Axiocam 503 Color digital camera (Zeiss).

7.7. FLUORESCENCE MICROSCOPY

Resting or 3h IgE-stimulated CCL2-eGFP BMMCs in complete RPMI medium were washed twice with PBS, resuspended in fresh medium ($0.5x10^6$/ml) supplemented with $MnCl_2$ and plated into microscope 8-chamber slides (Lab-Tek^R) that were coated o/n with 10 µg/ml fibronectin (Sigma Aldrich). 15 min later Brefeldin A (BFA) (eBioscience, 1000x solution) was added to the medium and the chamber was placed on a 37°C pre-warmed heating plate, and controlled 5% CO_2. After 15 additional minutes, 100ng/ml DNP and an equal number of freshly splenic and peritoneal cavity purified B cells were added in the slide. Phase-contrast images were recorded at the indicated time points within 5h of co-culture with in an inverted fluorescent microscope (Zeiss). Images were analysed by means of the ZEN software.

7.8. STATISTICAL ANALYSIS

In all experiments results are expressed as mean (+± SEM) of the indicated numbers of independent experiments. According to the number and type of groups in the analyses, 2-tailed paired or unpaired Student's *t test*, or one-way ANOVA with the Bonferroni correction as *post-hoc* analysis, were done to asses statistical significance with the GraphPad Prism 5 Software. A confidence level of 95% was used. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$.

8. APPENDIX

Animal models are used as potent predictors of human responses. However, it is fundamental to correlate data derived from animal studies with information about human to corroborate the most important elements of the model.

Using the algorithm CIBERSORT[329], relative proportions of 22 immune cells (LM22 signature) were determined on two different transcriptional profiles datasets (GSE87211 and GSE107499). Detailed information of immune cells are listed in *table 8*. In *figure 70A-B* immune cells infiltrating the colon of patients suffering from UC are reported: the non-inflamed intestine signature was compared with the one of the inflamed tissue. Figure *70C-D* analyse CRC patients comparing normal mucosa with the cancerous one. The first observation is that resting MCs infiltrating "normal" tissues became almost totally activated in inflammation and cancer. Naïve and memory B cells and differentiated PCs are also highly represented in the intestinal mucosa. Differently from MCs, these three populations are expanded in UC patients while are less depicted in cancer. This last result is in accordance with our previous data in which we showed accumulation of IgA^+ PCs in areas of low grade dysplasia and an extrusion of these cells from the adenoma[263]. Considering the relative relation between MC and PCs cells, in healthy portions of the colon there is an inverted correlation between activated MCs density and PCs. In UC tissues instead, the increase of activated MCs positively correlates with the increase of PCs. The trend is exactly the opposite in CRC, where the increase of MCs is followed by a decrease of PCs. This trend, according to the *p value* of the Spearman correlation, is also strongly statistically significant.

We can speculate that in patients suffering from acute inflammation, as we observed in our DSS mouse model, activated MCs are important for the expansion of IgA^+ PCs. In tumor condition, MCs expand and became activated and this may reflect in the accumulation of B cells in LNs directly draining the tumor where these cells can exert or immune-suppressive functions or promote immune-surveillance.

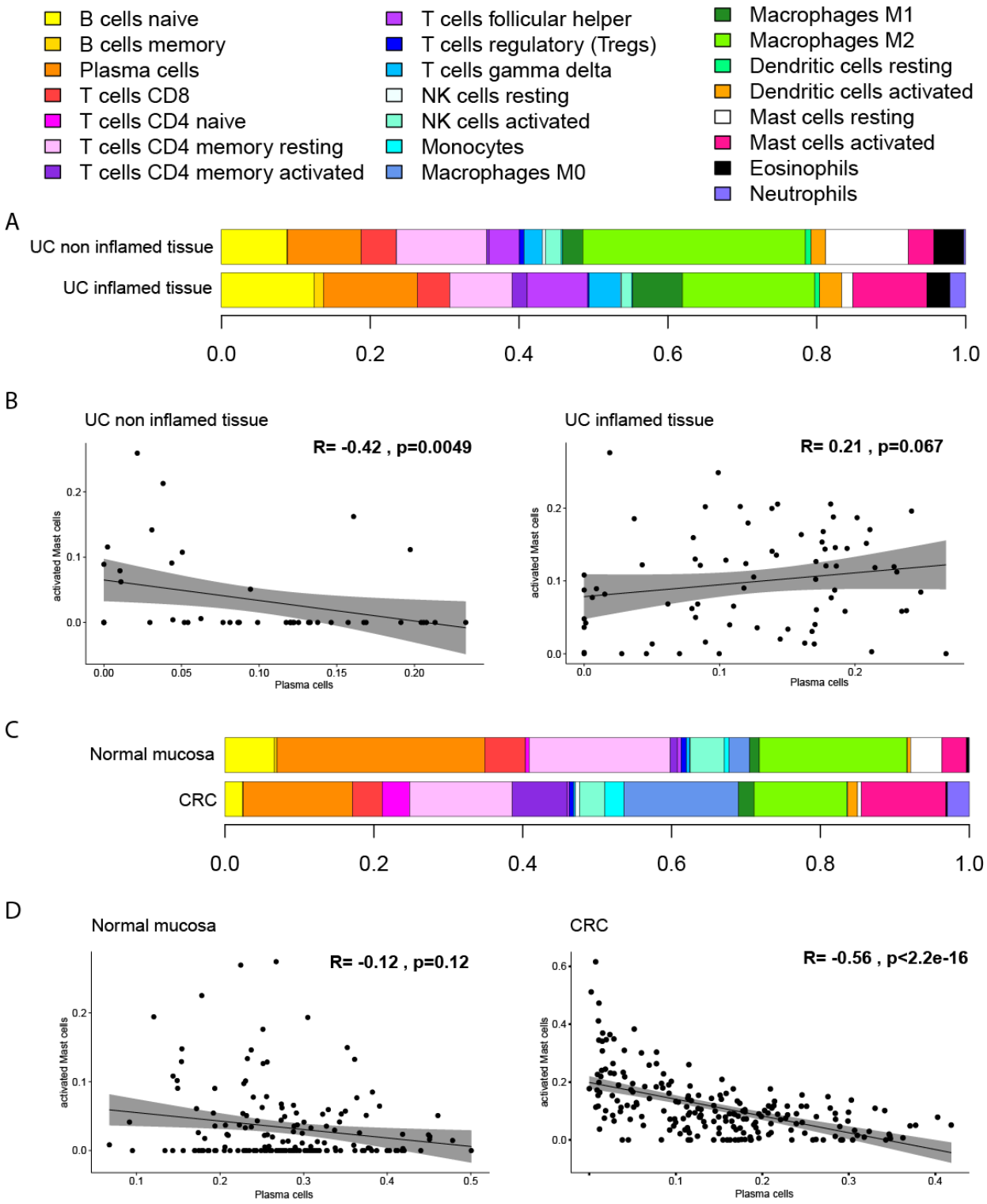


Figure 70: Relative proportions of 22 immune cell types in UC and CRC. Relative proportions of 22 immune cells determined on their gene expression profile in biopsies from UC **(A)** and CRC **(C)** patients. The correlation between the proportion of PCs and activated MCs was determined by Spearman correlation **(B** and **D)**.

Finally, with the aim of exploring the possibility whether CCL20 and CXCL13 are overexpressed in tumor dLNs also in human CRC, we used the GEO2R tool to compare the expression level of two cell lines derived from primary and metastatic CRC tumor cells (SW480 and SW620 cell lines). We chose this cell line pair as an accepted model for analyses concerning of CRC progression[330]. Interestingly, the expression of the two chemokines is higher in metastatic tumor cells, indicating that these two factors increase along with the progress of the tumor (*figure71*). This data suggests the importance of these two chemokines in the CRC setting and indicates that they can be considered important factors for the recruitment of B cells in metastatic CRC also in men.

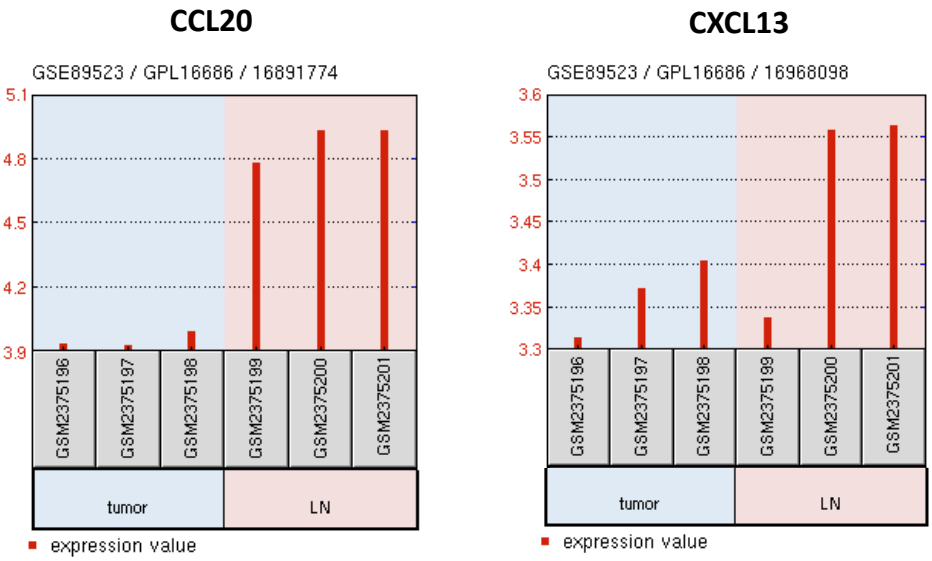


Figure 71: CCL20 and CXCL13 are more expressed in LNs metastasis compared to primary CRC tumor. CCL20 and CXLC13 expression was determined on GSE89523 dataset by using the GEO2R web tool, comparing SW480 and SW620 cell lines.

Table 8: Methods used for the determination of immune infiltrates

LM22 Cells	Cell Type Description	Cell Separation Method	Markers used	Purity
B cells	B cells naive	MACS® CD138 microbeads and CD19 microbeads	CD19+CD27-IgG/A-	Not stated
	B cells memory	MACS® CD138 microbeads and CD19 microbeads, then FACS	CD19+ CD27+	Not stated
PCs	Plasma cells	MACS® CD138 microbeads, then FACS	CD20+, CD138+ and CD19+	Not stated
CD8 T	T cells CD8	RosetteSep™ CD8+ T-cell enrichment cocktail, CD-8 subset	CD3, CD8, CD45RA	>90
CD4 T cells	T cells CD4 naive	Ficoll, then MACS CD4+ T cell isolation kit	CD4+	>98%
	T cells CD4 memory resting	Ficoll, then FACS	$CD45RO^{high}$	Not stated
	T cells CD4 memory activated	Ficoll, then FACS, then activated by anti-CD3 (plate-bound) + anti-CD28 (soluble)	$CD45RO^{high}$; CD69, CD25 for activation	>90%
	T cells follicular helper	Ficoll, then MACS CD4+ T cell isolation kit, then FACS	$CXCR5^{hi}$, $ICOS^{hi}$	>95%
	T cells regulatory (Tregs)	Ficoll-Hypaque, then MACS CD4+ T cell isolation kit, then FACS	CD4+ $CD25^{hi}$	>98%
Gamma delta T cells	T cells gamma delta	Ficoll, then FACS	Not stated	Not stated
NK cells	NK cells resting	RosetteSep™ NK-cell enrichment cocktail + CD2 Microbeads	CD56	Not stated
	NK cells activated	RosetteSep™ NK-cell enrichment cocktail + CD2 Microbeads + IL2 or IL15 for activation	CD56 + CD69	Not stated
Monocytes and Macrophages	Monocytes	MACS® CD14 Microbeads, monocyte subset	N/A	Not stated
	Macrophages M0	Differentiated from monocytes	None known; identified by morphology and phagocytic capability	Not stated
	Macrophages M1	Histopaque 1.077, then Miltenyi negative selection monocyte isolation kit and LS columns, then differentiated with 1% medium supplement nutridoma-HU + 100 nM M-CSF, then activated with 20 ng/ml IFN-g+ 100 ng/ml LPS	None known; identified by morphology and phagocytic capability	>97% (at monocyte stage)
	Macrophages M2	Histopaque 1.077, then Miltenyi negative selection monocyte isolation kit and LS columns, then differentiated with 1% medium supplement nutridoma-HU + 100 nM M-CSF, then activated with 20 ng/ml IFN-g+ 100 ng/ml LPS and 20 ng/ml IL-4	None known; identified by morphology and phagocytic capability	>97% (at monocyte stage)

Dendritic cells	Dendritic cells resting	Monocytes differentiated with 17 ng/ml IL4, and 67 ng/ml GMCSF	N/A	Not stated
	Dendritic cells activated	Monocytes differentiated with 17 ng/ml IL4, and 67 ng/ml GMCSF, then stimulated with 1 ug/ml LPS	N/A	Not stated
Mast cells	Mast cells resting	Ficoll of cord blood, then 100 ng/ml SCF + 10 ng/ml IL-10 + 5 ng/ml IL-6	N/A	95%
	Mast cells activated	Ficoll of cord blood, then 100 ng/ml SCF + 10 ng/ml IL-10 + 5 ng/ml IL-6 + IgE receptor activation	N/A	
Eos	Eosinophils	0.6% Dextran T500, then Percoll gradient (70%, 80%), then negative selection with MACS CD16 Microbeads	N/A	>97%
PMNs	Neutrophils	0.6% Dextran T500, then Percoll gradient (70%, 80%), then negative selection with MACS anti-CCR3 + anti-mouse IgG Microbeads	CD62L	>97%

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10. LIST OF PUBLICATIONS

Full papers

- Mion F., Vetrano S., Tonon S., **Valeri V**., Piontini A., Burocchi A., Petti L., Frossi B., Gulino A., Tripodo C., Colombo MP., Pucillo CE.
  Reciprocal influence of B cells and tumor macro and microenvironments in the Apc$^{Min/+}$ model of colorectal cancer.
  Oncoimmunology . doi: 10.1080/2162402X.2017.1336593
- Mion F., Tonon S., **Valeri V.**, Pucillo CE.
  Message in a bottle from the tumor microenvironment: tumor-educated DCs instruct B cells to participate in immunosuppression.
  Cell Mol Immunol. doi: 10.1038/cmi.2017.63

Conference proceedings

- B cell-MC crosstalk takes the stage in the gut: effector and regulatory functions of conventional B cells are coordinated by MCs
  Poster presentation at the EMBO Workshop To-B or not to-B: B-cells in health and disease; Girona (Spain), September 2017
- MCs and B cells bidirectional cross-talk: mutual support in homeostasis and inflammatory conditions
  Oral presentation at the 8th EMBRN International Mast cell and Basophil Meeting; Prague (Czech Republic), May 2017
- MCs and B cells bidirectional cross-talk: mutual support in homeostasis and inflammatory conditions
  Poster presentation at the International Retreat of PhD Students in Immunology; Naples (Italy), September 2016
- *Analysis of BAFF and APRIL expression by mast cells: novel actors in the B/MC cross-talk*
  Poster presentation at the *X National Congress SIICA - Italian Society of Immunology, Clinical Immunology and Allergology;* Abano Terme (Padua, Italy), *May 2016*

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